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Polymer Applications for Improved Biofuel Production from Algae

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Polymer Applications for Improved Biofuel Production from Algae

by

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Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

December 2011

Dedication

I dedicate this work to my parents, Steven Max Jones and Kimiko Wakiyama Jones, for their unwavering and unconditional support as I pursued my education goals. Mom and Dad, you have taught me that anything is possible if I put my mind to it.

Acknowledgements

I thank my advisor and mentor Dr. Martin Poenie for his guidance over the past six years. His skill and work ethic are a constant inspiration to me.

I thank my committee members, who lend their broad range of expertise to this research problem: Drs. Jerry Brand, Jennifer Brodbelt, George Georgiou, Krishnendu Roy, and Frank Seibert.

I have had the informal support of several outstanding *senpai* (Japanese term for senior student). Dr. James Beach gave me a foothold in polymer chemistry and conducted work during the formation of this project. Dr. Mark Fountain edited and commented on manuscripts, and offered his mass spectrometry experience. Dr. Schonna Manning brought a strong background in algal studies and phytochemistry, and has been a patient sounding board during the dissertation writing process.

I was fortunate to have the friendship and support of my student peers: Laura Christian, Kathryn Moncivais, Morela Montoya, Vaishnavi Narasimhan, Hassan Naqvi, Shubhankar Nath, and Sarah Tan.

I had the assistance of many brilliant and hard-working undergraduate students while conducting my experiments: Christine Barnes, Jishnu Das, Eric Jones, Han Lee, Andrea Middleton, Thanh-Tu Nguyen, Nicholas Reyna, James Wang, Andrew Widener, and Benjamin Wolfson.

The engineering team at the Center for Electromechanics, led by Dr. Robert Hebner and Michael Werst, have been wonderful collaborators in the pursuit of algal biofuel commercialization. This work has been supported by CEM and OpenAlgae, LLC.

Polymer Applications for Improved Biofuel Production from Algae

Jessica Naomi Jones, Ph.D.

The University of Texas at Austin, 2011

Supervisor: Martin Poenie

Biofuel is a renewable and sustainable energy source with near-neutral carbon footprint. Algae are an ideal feedstock for biofuel production because they reproduce quickly and have high oil. Algae can be cultivated in non-arable land, and would not impact the food supply. Unfortunately, processing algae into biofuel is more expensive than land crops due to the large volumes of dilute algal suspension that must be harvested and concentrated. In order to improve algae-based biofuel economics, resins were developed that reduce costs associated with water pumping and transport. Hydrophobic resins were developed for binding oil out of an algal suspension so that the residual biomass could be recovered without solvent contamination. Binding behavior displayed lipid species specificity, and binding capacity was improved by ethanol treatment of the biomass. Algae was harvested by binding to anion exchange resin and directly converted into biodiesel. One-step, room temperature *in situ* transesterification of algae yielded nearly as much biodiesel as two-step, heated transesterification of dried biomass. Elution with transesterification reagent also regenerated the resin for subsequent algal binding. Functionalized resins were developed with high algal binding capacity at neutral pH. Binding was easily reversed, as treatment with buffer with pH higher than pKa of the resin functional group removed the algae and regenerated the resin for subsequent use.

The resin bound 10% of its weight in algae and released it as a 100-fold concentrated suspension. The polymers developed can be scaled up for commercial processes and reduce algal harvesting and concentration costs.

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Chapter 1: Introduction

This dissertation describes the development of polymers used in the production of biofuel from algae. Algae are a renewable and sustainable feedstock, and can potentially address current and future energy demands. Unfortunately, the high cost of algae-derived biofuel prevents its widespread adoption. The goal of the study is to explore new methods for decreasing algae-based biofuel production cost so that it can be competitive with petroleum fuel.

Chapter 2 is an introduction to algal biofuel as an alternative energy source. The advantages of algae over other plant feedstocks and the growth and oil production in algae are discussed. Recent literature has been reviewed in order to identify areas critical to algal biofuel viability. Current processing methods are surveyed to provide a baseline for comparison with the new methods developed herein.

Since the survey of lipid content is vital to screening algal species, methods for lipid analysis are described in Chapter 3. Procedures for laboratory-scale algal lipid extraction are compared. Chromatographic and spectroscopic analytical techniques, and their application to algal lipids, are described. Our methods for algal lipid extraction and analysis are discussed within the context of the review.

The project investigated ways to improve the processing of algal biomass into crude oil products. Towards this end, three aims were proposed for the study: (1) the development of methods for concentrating and dewatering algae, (2) the development of a one-step method for *in situ* generation of biodiesel, and (3) the development of an algal lipid extraction method that eliminates solvent contamination of residual biomass. These aims addressed key areas of optimization needed for algal processing after culture: biomass harvesting, biodiesel synthesis, and oil extraction.

The development and application of hydrophobic resins is described in Chapter 4. A polymer-based oil separation method would improve process economics by reducing extraction solvent use and residual biomass contamination. Oil extraction was a more complicated problem than simple liquid phase separation of cell components. Algal lipids reside in intracellular compartments that are surprisingly difficult to liberate from the cellular biomass, even after the cell walls have been mechanically compromised. We were able to recover nearly 100% of an oil emulsion in control studies, and 2% of the resin weight as oil from the algal suspension. Addition of hydrophobic or hydrophilic resin surface groups affected lipid species selectivity. Dilute ethanol was also used to improve oil recovery by transferring oil through the suspension to the hydrophobic resins.

In Chapter 5, anion exchange resins were used for algal harvest by binding algal biomass out of a flowing suspension. Concentrating algae requires energy to process large growth media volumes, and harvesting at high flow rates is difficult. A polymer-based method simplifies algal harvest and biodiesel conversion into a single step; the concentrate of resin-bound algae was easily dewatered, and immediately treated with transesterification reagents in order to generate biodiesel. Water removal from the biomass was critical, since its presence inhibits transesterification of triacylglycerol (oil) into fatty acid methyl esters (biodiesel). We found that room temperature transesterification of algae bound to the resin yielded nearly as much biodiesel as heated transesterification of dried algal biomass.

Chapter 6 describes the development of functionalized resins for harvesting algae out of suspension. The binding of algae to fixed-charge anion exchange resins suggested the possibility of reversible binding to weak-base ion exchangers as a function of pH. Accordingly, a series of monomers bearing weakly basic amines of different pKa values were synthesized and tested for binding capacity and the ease of algal removal off of the

resin. The test regime involved measuring the binding of algae at a lower pH where the amine was protonated and positively charged, followed by raising the pH to generate a neutral amine that no longer bound algae. Through these tests, polymers were identified that could bind 10% of their weight in algae and release essentially 100% of the bound algae when the pH was raised. Elution with smaller volumes of basic buffer yielded a concentrated algal suspension. These resins could serve as the foundation of an inexpensive harvesting system that does not entail pumping large volumes of water.

Potential cost savings from the use of low-cost polymers for harvest and oil extraction advances the future adoption of algae-derived biofuel. Chapter 7 provides concluding remarks on the project and discussion of the future algae-based biofuels. The primary finding of the study was that a functionalized resin could bind algae with high capacity into a highly concentrated algal biomass. The next step is scale-up to commercial harvest levels, possibly by formulating surface coatings for current pond culturing implements.

Chapter 2: Survey of algae-based biofuel production

2.1. The need for an sustainable, renewable energy source

Sustainable fuels must be commercially developed in order to meet current and future energy needs (Hossain et al. 2008). The predominant alternative energy modes are hydroelectric, biomass, geothermal, solar and wind (Csavina 2008). Of these modes, biofuels, generated from biomass, are an attractive option since they can be used in existing engines and infrastructure (Scott et al. 2010). Assuming optimized production practices, some government studies have shown that biofuels can have positive energy balances, yielding greater energy output than required to produce it (Schnepf 2005), and that they release less air pollutant than petroleum fuel per unit energy (Hill et al. 2006). Biofuel can be produced in the form of methane, generated by anaerobic digestion (Hossain et al. 2008); conversion of cellulose into ethanol (Huber et al. 2006), photobiotic generation of hydrogen (Hossain et al. 2008); and conversion of lipids into biodiesel (Chisti 2007).

Plants provide a renewable source of biomass for biofuel production. Through photosynthesis, plants can store solar energy in molecules that can be converted into biofuel (Rittmann 2008). Biofuels from sugarcane, palm oil, sugar beet, rapeseed, soybean, wheat, and corn are already extensively used in the United States, Brazil, southeast Asia, and Europe (Stephens et al. 2010). However, while agricultural crops have been used successfully for local production, the generation of enough crop-based fuels for commercial demand would divert more than half of the food supply, and require an unrealistic proportion of land mass (Scott et al. 2010).

Algae are the most sustainable feedstock for biofuel. Certain algae can replicate quickly, sometimes doubling in biomass every day, and during exponential growth can

even double within 3.5 hours (Vijayaraghavan and Hemanathan 2009). Algal cultivation is not limited to arable land, and growth facilities have been built in deserts around the world (Ben-Amotz 2008). Most importantly, cultivation of algae does not divert from the food supply (Chisti 2008). While it has been projected that 61% of agricultural land in the United States would be required to farm enough corn to displace petroleum fuels, algae would only require 3% of the land (Chisti 2008). As a comparison, land requirements for supplying one terawatt of energy per year were calculated for corn-based bioethanol and algae-based biodiesel in Figure 2.1. Corn cultivation for bioethanol would require 62% of the landmass of the state of Texas. However, given the high energy density of biodiesel (comparable to petroleum-derived diesel) and assuming a highly productive algal species, only 10% of Texas landmass would be required to supply 1 terawatt[1]. Note that current global demand for energy is approximately 13 terawatts (4.16×10^{14} kilojoules per second) per year. For these reasons, algae are better suited to address energy demand than other plant feedstocks.

This chapter is a survey of algae-derived biofuel and production process. Given that alternative energy development is a significant scientific concern, the potential and benefits of algae are explored. For in-depth analysis of the petroleum fuel crisis—the depletion of fossil fuel reserves in the face of increasing worldwide energy demand—and the agricultural, geopolitical, and environmental effects of energy dependence, comprehensive reports from world policy groups, such as the United Nations Environmental Programme (UNEP) (Bringezu et al. 2009) and the International Energy Agency (IEA) (OECD 2010) are referenced.

[1] Fuel and land productivity based on Rittmann (2008) and Grossman (2011).

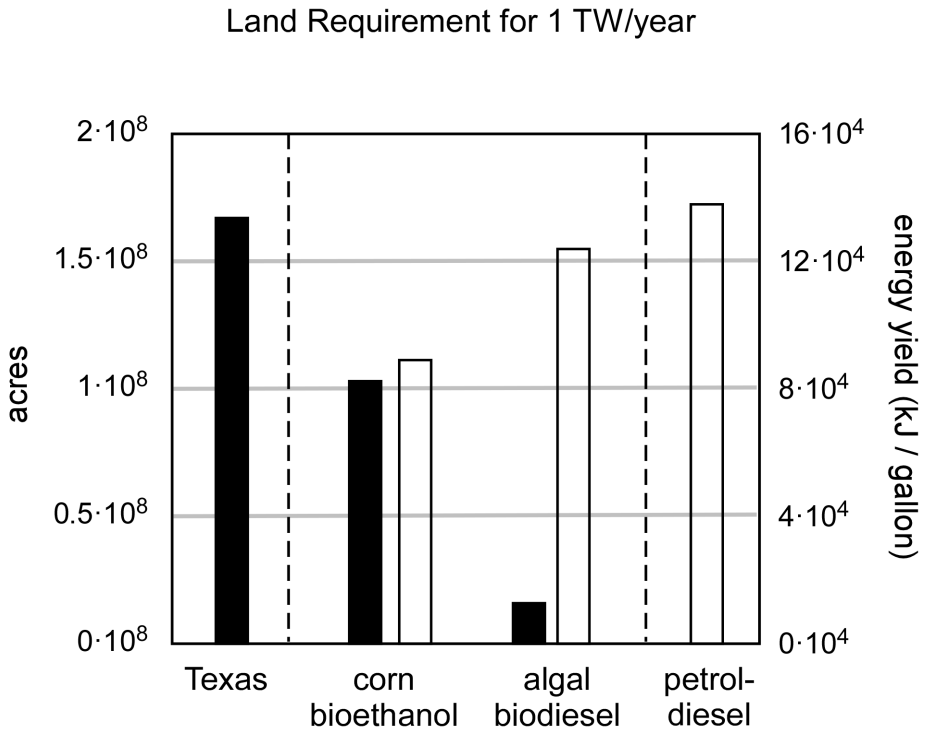


Figure 2.1: Comparison of cultivation acreage required for one terawatt of energy per year between corn bioethanol and algal biodiesel.

Cultivation land required to produce one terawatt of energy per year (solid bars), and energy density in one gallon of fuel (hollow bars) is compared between corn-based bioethanol and algae-derived biodiesel. For scale, the required landmasses are compared to the non-water landmass of the state of Texas (167 million acres). The energy densities of the biofuels are compared to that of petroleum-derived diesel. Corn bioethanol projection is based on Rittmann (2008), algal biodiesel projection is based on Grossman (2011).

While organisms such as bacteria, fungi, and macroalgae have also been considered for biofuels, we narrow the focus to microalgae and will refer to this group as simply, "algae". Algae are a diverse group of over 25,000 known species (Raja et al. 2008). Biofuel research is centered on green alga (Chlorophyta) species, not only because of oil content, but also because of worldwide availability, fast growth rates, and ease of laboratory manipulation (Hu et al. 2008). Many of the species named in this chapter are green algae, though some oleaginous diatom species are mentioned due to their oil content and prevalence in aquaculture production. Algae referenced in this chapter are listed in Appendix.

2.2. ALGAE, THE ENVIRONMENTALLY-FRIENDLY FUEL SOURCE

2.2.1. Carbon fixation

Increased carbon fixation by plants can offset the greenhouse effect caused by elevated atmospheric carbon dioxide levels (Bekkering 1992). Carbon fixation is driven by photosynthesis, which converts gaseous carbon dioxide into glucose and oxygen. Algae are prodigious carbon fixers, and as the foundation of marine ecosystems, already account for half of the total global carbon fixing activity (Leon-Banares et al. 2004). Species of microalgae have been screened for higher carbon fixation capacity, since increased carbon fixation activity does not necessarily result from increased carbon availability (Murakami and Ikenouchi 2004).

Carbon fixation gives algae-derived biofuel a near-neutral carbon footprint. Given that algal carbon composition is nearly half of its dry weight, production of 100 tons of algal biomass would fix approximately 183 tons of carbon dioxide (Chisti 2008). Using

carbon dioxide waste would not only be environmentally beneficial, it would improve process economics (Kadam 2002). Supplying carbon dioxide is expensive for algal cultivation, accounting for 20-30% of growing costs in some studies (Huber et al. 2006). The use of flue gas as a carbon dioxide source has been demonstrated with *Chlorella sp*. cultivation within a photobioreactor (Doucha et al. 2005) and in pilot-level reactor studies using *Dunaliella sp*. (Vunjak-Novakovic et al. 2005).

2.2.2. Wastewater remediation

Water supply purification and safe disposal of contaminants are constant public health issues. Algae have been used to remediate wastewater in an ecologically-friendly manner. They can treat toxic streams by binding heavy metals and pathogens, and degrading organic pollutants (Munoz and Guieysse 2006) (Gavrilescu and Chisti 2005). Growing algae in freely available sewage recycles the nitrogen and phosphorus content of the water (Rodolfi et al. 2009), while simultaneously reducing inputs associated with nutrient supplementation (Sawayama et al. 1995). For example, *Chlorella sp*. has been grown at a wastewater facility, where it removed ammonia from sewage and fixed carbon from flue gas (Yun et al. 1997), and *Botryococcus braunii* reduced inorganic nutrients in sewage and removed toxic metals while producing oil (Sawayama et al. 1995).

2.3. ALGAE AS OIL PRODUCERS

In many algae, excess photosynthesis product can be converted into lipids as a long-term storage product (Dunahay et al. 1996), which can be converted into biofuel of high energy density. Lipids are water-insoluble macromolecules composed of fatty acids and their derivatives; examples of plant lipid classes are shown in Figure 2.2.

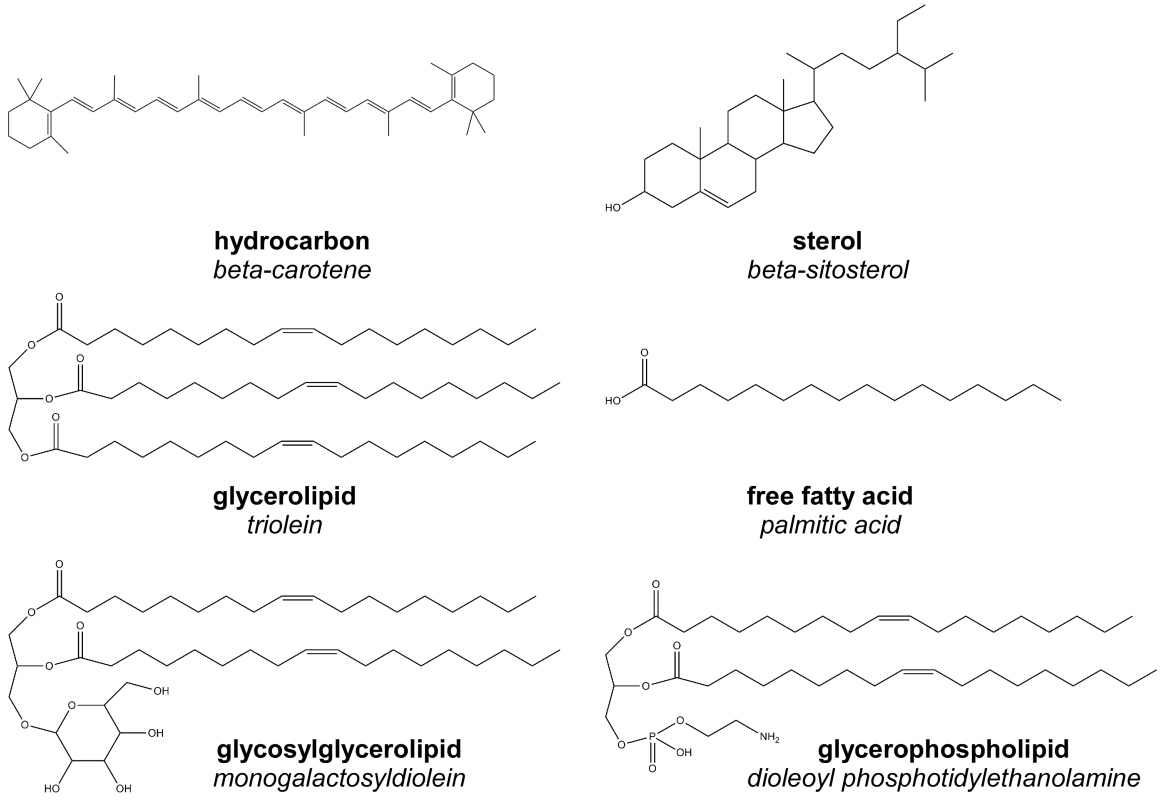


Figure 2.2: Examples of lipid classes found in plants.

Hydrocarbons consist entirely of hydrogen and carbon atoms. Sterols are a subgroup of steroids, which contain a specific arrangement of four cycloalkane rings. Glycerolipids are composed of a glycerol esterified to fatty acid chains. Fatty acids are hydrocarbon chains that terminate with a carboxylic acid. Glycosylglycerolipids are composed of a glycerol esterified to fatty acid chains and a sugar group. Glycerophospholipids are composed of a glycerol esterified to fatty acid chains and a phosphate group. Lipid classes are shown in order of increasing polarity.

In order to be a competitive oil source, lipids must comprise 50-60% of the biomass weight (Liu et al. 2008). Biofuels have focused on high oil content plants such as rapeseed, *Jatropha*, and soybean, which have oil productivities of 40%, 30%, and 20% of dry weight, respectively (Scott et al. 2010). Algae have also been investigated as a plant oil source, as they are capable of both high biomass production rate (Li et al. 2008), and high oil productivity, ranging from 30 to 70% of dry weight (Chisti 2007).

Algal cultivation also yields greater energy return than land plants. Algae have a photosynthetic efficiency[2] of 10-20%, compared to 0.5% for higher plants (Li et al. 2008), and can produce lipids with thirty times more efficiency than higher plants (Sorguven and Ozilgen 2011). Algae would produce the most oil per landmass, yielding 250 times more oil per acre than soybean (Hossain et al. 2008).

2.3.1. Triacylglycerol is the feedstock of biodiesel production

Lipid biosynthesis in algae is believed to be similar to that of higher plants (Hu et al. 2008). Briefly, fatty acid acyl chains are assembled in the chloroplasts by successive condensation reactions beginning with the substrate acetyl CoA, as shown in Figure 2.3 (Hu et al. 2008). Algae can produce the same variety of fatty acids as higher plants, including both saturated (myristoyl, C14:0; palmitoyl, C16:0; steroyl, C18:0) and unsaturated species (oleoyl, C18:1; linoleoyl, C18:2) (Ratledge 1993). The fatty acids are sequentially transferred onto a glycerol backbone to form triacylglycerols, which collect as lipid bodies in the cytoplasm (Hu et al. 2008).

[2] Defined in Li et al. (2008) as the conversion of solar energy into biomass energy, in units of watts per square meter.

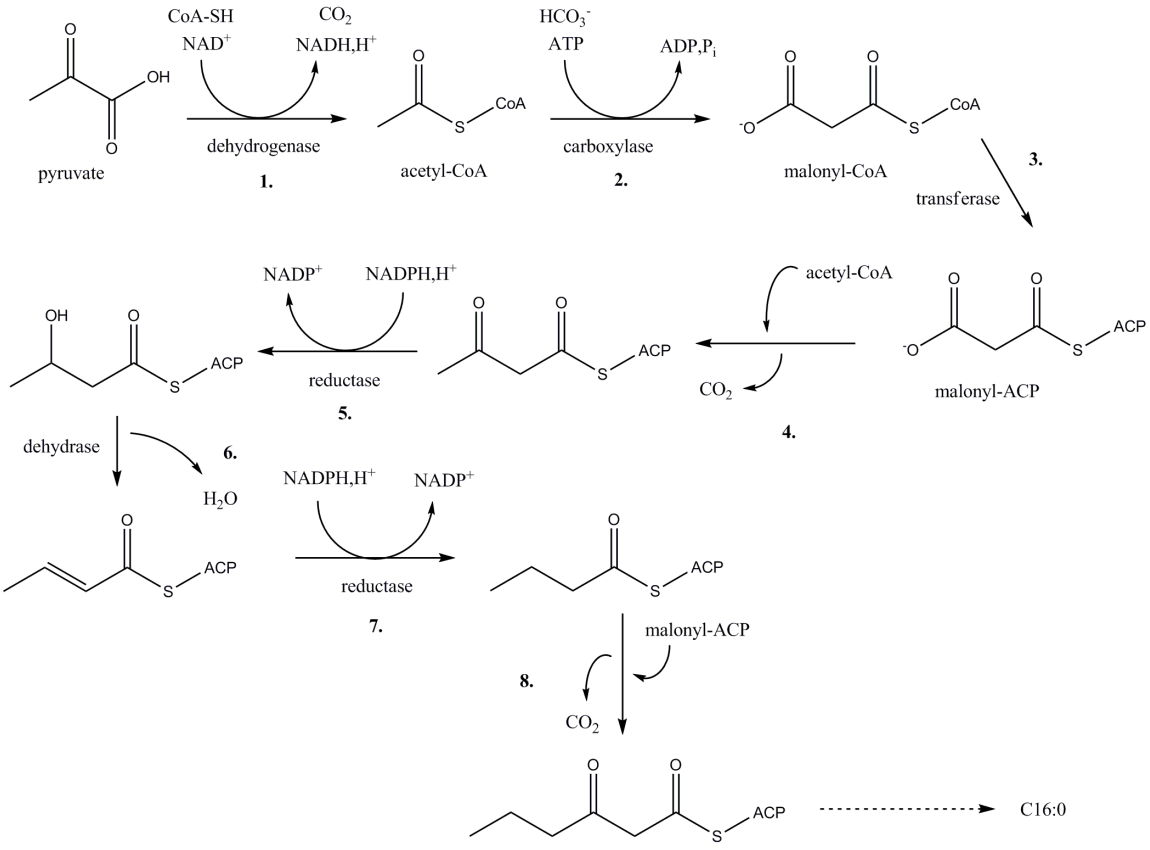


Figure 2.3: Fatty acid synthesis from pyruvate, generated by photosynthesis/glycolysis or fermentation.

(1) pyruvate is oxidized and converted into acetyl coenzyme A (acetyl CoA) by pyruvate dehydrogenase, (2) acetyl CoA is carboxylized and converted into malonyl CoA by acetyl CoA carboxylase, (3) malonyl is transferred from CoA to acylcarrier protein (ACP) by malonyl CoA:ACP transferase, (4) condensation of acetyl CoA and malonyl-ACP extends the molecule by two carbons, (5) molecule is reduced to alcohol by *beta*-ketoacyl-ACP reductase, (6) molecule is dehydrated to form an alkene by *beta*-hydroxylacyl-ACP reductase, (7) the double bond is reduced by enoyl-ACP reductase, (8) the cycle to add two carbon units begins again by condensation with malonyl ACP, (9) the molecule is built up to palmitic acid (C16:0). Adapted from Nelson and Cox (2000) and Hu et al. (2008).

Algae have evolved mechanisms to determine whether to shift metabolic activity towards growth or lipid accumulation. Enhanced lipid production results in triacylglycerol accumulation for energy storage. By contrast, glycosylglycerol and phosphoglycerol, the chloroplast and plasma membrane lipids, are synthesized during cell doubling (Hu et al. 2008). Thus lipid content and composition varies with the cell growth cycle, with triacylglycerol accumulating during stationary phase (Hu et al. 2008).

2.3.2. Other important lipid classes

Algae can produce lipid types other than triacylglycerol that are relevant natural products. Hydrocarbons, nonpolar lipids comprised of linear or branched alkane groups, can be converted by cracking or pyrolysis into short chain-length linear alkanes suitable for combustion. Commonly referred to as waxes, hydrocarbons are synthesized and maintained near the cell membrane surface; their synthesis is limited to species of algae, bacteria, and fungus (Tornabene 2008). Algae normally produce a limited hydrocarbon supply (less than 5% of dry weight) because of the high metabolic requirement for its synthesis, though exceptions have been explored. Most notably, *Botryococcus braunii* secretes large amounts of hydrocarbon (up to 80% of total dry weight) into its extracellular matrix (Ben-Amotz et al. 1985) (Banerjee et al. 2002) (Metzger and Largeau 2005). Fuels have been generated from *Botryococcus braunii* by thermoliquefaction of algal biomass and catalytic cracking of crude oil extract (Banerjee et al. 2002). Unfortunately, despite the promise of easily accessible fuel from secreted hydrocarbon, *Botryococcus braunii* reproduces slowly and is notoriously difficult to culture, limiting efforts for scale-up.

Current large-scale algal cultivation is for high-value products such as long-chain poly-unsaturated fatty acids (PUFA) (Lebeau and Robert 2003). The diatoms

Phaeodactylum tricornutum and *Monodus subterraneus* are cultivated for eicosapentanoic acid (EPA, C20:5 ω-3) (Cartens et al. 1996; Belarbi et al. 2000), and *Isochrysis* sp. for docosahexanoic acid (DHA, C22:6 ω-3) (Liu and Lin 2001). Both are essential *omega*-3 fatty acids, and have been shown to have cardiovascular and prenatal health benefits (Cartens et al. 1996) (Pyle et al. 2008). Algae are also rich in pigments, lipids that have high anti-oxidant and anti-inflammatory properties, such as *beta*-carotene and astaxanthin (Spolaore et al. 2006). The market for these algal lipids in health and cosmetic products has grown significantly, and demand for these high-value products offsets high production and extraction costs.

2.3.3. Improving lipid production capacity

Algae enhance lipid production in response to environmental stress, though the specific pathways responsible are not well characterized (Tornabene et al. 1983). Temperature, irradiance, and nutrient availability modulate lipid composition and content (Rodolfi et al. 2009). Optimal growing temperature is within 20-30 ºC (Chisti 2008). At low temperatures, algae increase production of unsaturated fatty acids in order to maintain membrane fluidity (Renaud et al. 1995) but this does not necessarily lead to increased total lipid content. In contrast, several species have been identified as high biomass and lipid producers at temperatures above 30 ºC, including *Chaetoceros muelleri* and *Cyclotella cryptica* (Sheehan et al. 1998). Increased irradiance causes photo-oxidative stress, which can increase lipid production (Hu et al. 2008). Adaptation to high and constant illumination resulted in four-fold increase of hydrocarbon production in *Botryococcus braunii* (Banerjee et al. 2002). *Beta*-carotene production was induced in *Dunaliella salina* by excess light after a normal growth stage (Hejazi and Wjiffels 2004). Temperature and light intensity modulation has been used to influence lipid

accumulation, though tolerance levels must be explored to ensure the long-term health of a large culture.

The "lipid trigger", or the switching of algal metabolism to a phase of high lipid accumulation, has been an area of intense study in biofuels (Sheehan et al. 1998). Essential nutrients required for algal growth, including nitrogen, phosphorus, iron and silicon, are deprived in order to improve neutral lipid production by shifting metabolic activity away from membrane lipid formation (Chisti 2007). Nitrogen starvation is a routine procedure for inducing neutral lipid accumulation, as demonstrated in *Chlorella sp*. (Illman et al. 2000), *Nannochloropsis sp. (Ben-Amotz et al. 1985; Rodolfi et al. 2009), Neochloris oleoabundans* (Tornabene et al. 1983; Li et al. 2008), *Scenedesmus sp*. (Rodolfi et al. 2009), and *Tetraselmis suecica* (Rodolfi et al. 2009). These species are particularly noteworthy because not only do they accumulate high oil content, their biomass productivity remains adequate for commercialization, whereas many species have been found to only produce oil at the expense of biomass production. Higher lipid productivity has also been induced by phosphorus limitation in *Monodus subterraneus* (Khozin-Goldberg and Cohen 2006), sulfur limitation in *Chlorella sp*. (Otsuka 1961), and silicon limitation in *Cyclotella cryptica* (Dunahay et al. 1992).

Heterotrophic algal species can produce oil through fermentation of glucose or another carbon source in the absence of sunlight (Li et al. 2008). Simplified autotrophic and heterotrophic pathways for lipid production are shown in Figure 2.4. While more expensive than outdoor algal culture, high heterotrophic productivity justifies its cost (Ratledge 1993). High oil content has been demonstrated in *Chlorella protothecoides* (Miao and Wu 2006), and species of *Crypthecodinium* and *Schizochytrium* have been grown heterotrophically for DHA (Spolaore et al. 2006). Reactor fermentation yields biomass concentrations greater than 100 g/L with 85% oil composition, compared to

autotrophic growth yield of 1 g/L with 5-30% oil content (Grossman 2011). However, oil production by heterotrophs is ultimately a secondhand utilization of solar energy, since the carbon source is usually supplied by autotrophic plants. While ideally, carbon waste products should be used (Chisti 2007), they must be in a form that can be utilized by the algae.

Algae have been genetically modified in order to improve lipid production (Dunahay et al. 1996), and to create transgenic lines for containment and controlled growth within photobioreactors (Leon-Banares et al. 2004). Genetic work has been conducted predominately with the model algae *Chlamydomonas reinhardtii*, *Volvox carteri*, and *Phaeodactylum tricornutum* (Beer et al. 2009). By elucidating the poorly understood mechanisms that shift carbohydrate production to that of fatty acids, algae could be engineered for higher oil content and reduce the scale biomass cultivation. For example, the activity of the enzymes nitrate reductase and acetyl-CoA carboxylase have been identified as genetic targets, implicated by observed increased lipid production in cases of nitrogen and silicon deficiency, respectively (Dunahay et al. 1992). While advances have been made in developing the molecular toolkit and understanding of algal genetic mechanisms, the research has not yet reached a point where it can translate to biofuel technology (Vasudevan and Briggs 2008).

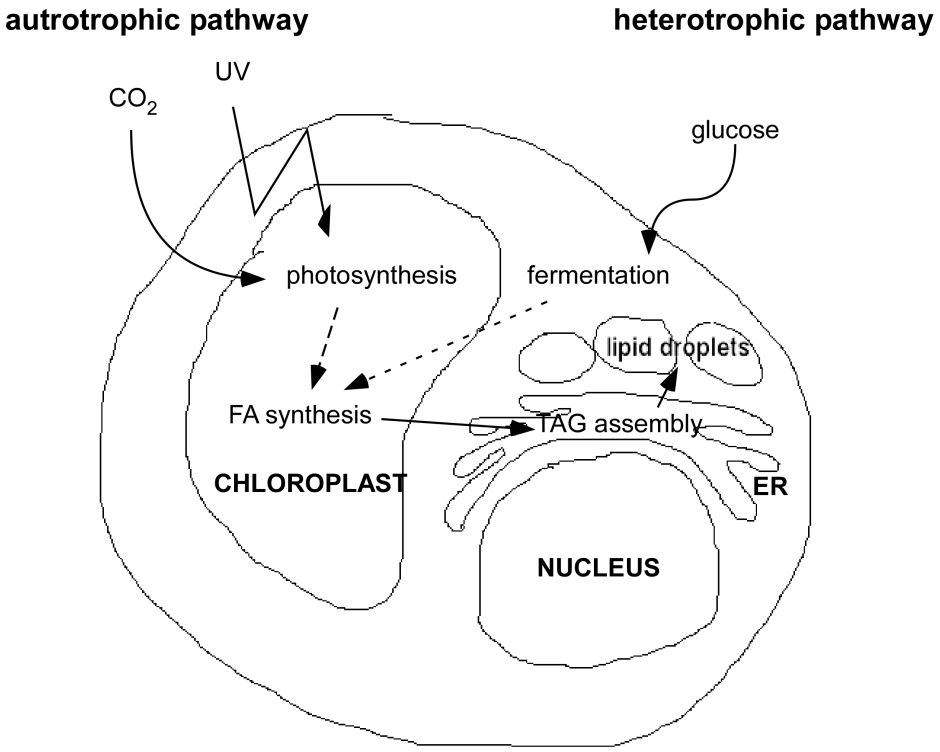


Figure 2.4: Autotrophic and heterotrophic pathways for lipid synthesis.

In the autotrophic pathway, photosynthesis in the chloroplast is driven by light and carbon dioxide. Conversely, glucose is fermented in the cytoplasm in the heterotrophic pathway. Photosynthesis and fermentation products are used for fatty acid (FA) synthesis, also in the chloroplast, though some species of algae may synthesize fatty acids in the cytoplasm. Fatty acid is assembled into triacylglycerol (TAG) in the endoplasmic reticulum (ER), which is exported into the cytoplasm as lipid droplets. Adopted from Beer et al. (2009) and Scott et al. (2010).

2.4. HISTORICAL RESEARCH PERSPECTIVE OF BIOFUELS FROM ALGAE

The use of algae as a fuel source was first demonstrated in 1960 by Goleuke and Oswald (Benemann et al. 1982). Interest was again renewed in the 1970s, coinciding with increased petroleum fuel costs. Most notable of these efforts was the Solar Energy Research Institute (SERI)'s Aquatic Species Program (ASP), a program of the United States Department of Energy (DOE) (Sheehan et al. 1998). ASP screened high oil-producing algal species, developed cultivation scale-up models, and assessed biodiesel feasibility. Unfortunately, algae-derived biofuels were considered to be too difficult to pursue at the time, and research efforts were instead diverted to corn bioethanol.

Within the past decade, algae have again become an area of research interest, with government support from DOE and the Defense Advanced Research Project Agency (DARPA) (Pienkos and Darzins 2009). Enactment of the Energy Independence and Security Act (2007) and American Recovery and Renewal Act (2009) highlighted national concern in sustainable energy sources. Algae-based biofuel has fueled a burgeoning commercial industry, often working in collaboration with academic and national laboratories.

2.5. ALGAE-BASED BIOFUEL PRODUCTION PROCESS

Algae have been cultivated on the commercial scale as high-value nutrient feedstock; unfortunately, there are currently no large-scale commercial algal biofuel production facilities (Sander and Murthy 2010). As stated by Hu et al. (2008), "while the concept of using microalgae as an alternative and renewable source of lipid-rich biomass feedstock for biofuels has been explored over the past few decades, a scalable,

commercially viable system has yet to emerge." Based on current methods, large-scale algal biofuel production is at the best marginally feasible (Scott et al. 2010). In an early study of biofuel economics, Benemann et al. (1982) assessed costs based on a production scenario of 67.5 metric tons per hectacre per year, with algae of 40% lipid content, yielding 162 barrels of oil per hectacre per year. Assumptions included earthen raceway pond with paddles for growth, harvesting by bioflocculation and centrifugation, and wet solvent extraction of oil. Conservative and optimistic costs[3] were estimated for both flue gas and pure carbon dioxide supplementation cases. Table 2.1 summarizes estimated costs, scaled for inflation into 2011 U.S. dollars. On average, harvesting and oil extraction equipment contribute to nearly 30% and 10% of total capital costs, respectively. The resulting price per gallon of oil ranges between three to seven dollars. While some cases are on par with the current price of gasoline, note that these cases assume optimal growth conditions.

Grima et al. (2003) observed that, "the downstream recovery of algal products can be substantially more expensive than the culturing of the alga". Improvements should be made at the different production stages so that algal biofuels can be realized. The following section examines the current methods used for biodiesel production from algae, so that areas for development can be identified. As shown in Figure 2.5, production can be divided into cultivation of algae, biomass harvest, oil extraction, and biodiesel synthesis.

[3] Assuming nutrient recycling and high photosynthetic efficiency.

	Conservative		Optimistic	
	flue gas	pure CO_2	flue gas	pure CO_2
total capital costs	$103,883	$92,850	$64,867	$57,131
harvest equipment	*26.6%*	*29.8%*	*26.1%*	*29.7%*
extraction equipment	*8.3%*	*9.3%*	*13.3%*	*15.1%*
yearly operating costs	$47,497	$43,093	$24,232	$22,903
cost of barrel	$296	$268	$151	$142
cost per gallon	$6.88	$6.23	$3.52	$3.30

Table 2.1: Conservative and optimistic algal biomass production costs.

Estimates with either recycled flue gas or pure carbon dioxide supplementation, based on Benemann et al. (1982), corrected for inflation. Production assumes 67.5 metric ton biomass / ha / yr with 40% lipid content.

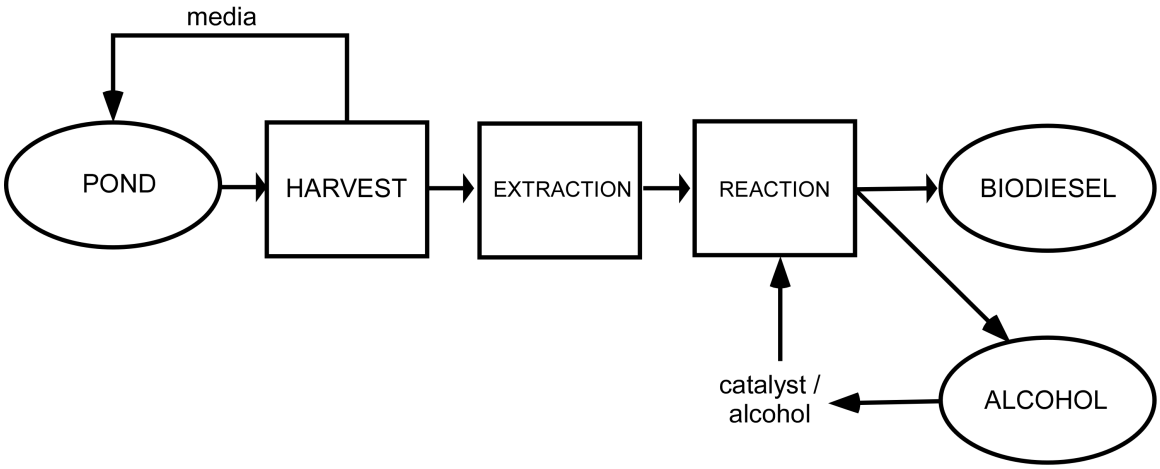


Figure 2.5: Process flow diagram for biodiesel production from algae.

Algae are cultivated on a large scale (pond) and the biomass is harvested from the growth media and concentrated (harvest). Oil is separated from the residual biomass (extraction) and converted into biodiesel with addition of catalyst and alcohol (reaction). The product is separated (biodiesel) and the reagent is distilled for reuse (alcohol).

2.5.1. Commercial-scale alga cultivation

The production of algae is currently 5-7 times more expensive than that of land-based crops (Huber et al. 2006). This may seem initially surprising; such a simple microorganism would presumably have a small agricultural footprint, especially considering how easily algae proliferate in open water. Unfortunately, successful conversion of algae into biofuel feedstock on the commercial scale is difficult.

Examination of process economics indicate that commercial feasibility depends on high biomass doubling rate, high algal oil content, and minimal growth demands (Chisti 2008). The fulfillment of all three criteria at once has been a challenge, as the achievement of one requires the tradeoff of another. Because of the small size of algae, several micrometers in diameter, high biomass production rates are required in order to

generate enough material to satisfy energy demand. However, lipid accumulation by algae tends to be inversely proportional to reproductive growth rate (Metting 1996). Increased lipid content is often triggered by stress conditions, and is more easily demonstrated on laboratory-scale culture where the environment is tightly controlled. In practice, high oil producing species tend to reproduce slower and are outcompeted by low oil content species. In addition, successful competitors thrive on whatever nutrient is available, while highly productive, high oil content algae require increased growth inputs.

In order to minimize cultivation costs, natural sunlight must be employed. When artificial lighting had been used to cultivate algae indoors, electricity accounted for over 90% of total production costs (Beal et al. 2011). However, using natural light results in less control over temperature, light intensity, and light duration, and the health of the culture is subject to seasonal and geographical variations (Li et al. 2008). In addition, as cultivation volumes increase, problems with light delivery arise that are not seen on lab-scale (Grossman 2011). First, light absorbance decreases with culture depth, so that algae beneath the culture surface receive less light. Second, high light intensity can exceed the threshold of light that the algae at the surface can safely absorb. In order to avoid damage of photosynthetic mechanisms, algae must dissipate excess light energy as heat. Thus, only a fraction of solar energy is actually used to generate oil. Commercial-scale algal cultivation must be optimized to provide adequate light access while maintaining low operating costs.

2.5.1.1. Raceway ponds

Outdoor growth ponds are already in use around the world for nutraceutical production and aquaculture. The structure is commonly a long closed-loop raceway design 10-300 meters in length, with shallow depth up to 100 centimeters (Ben-Amotz

2008). A simple raceway pond design is shown in Figure 2.6a. Raceway ponds must be situated for optimal lighting and aeration. Primary operational cost involves circulation and aeration of the pond, by paddles, pumps, or airlifts (Sander and Murthy 2010). The pond may be protected from the environment, either lined with plastic, concrete, or clay, or enclosed within a structure.

While relatively inexpensive to operate, raceway ponds face several challenges. The primary concern is that maintenance of an axenic culture is nearly impossible since the pond is open to the environment (Rodolfi et al. 2009). A contamination event resulting in a culture crash would require expensive draining and cleaning of the pond. Since algae are often cultivated in non-arable desert land, water evaporation is also a problem (Sander and Murthy 2010). An estimated 23 trillion gallons of water are evaporated from desert algal cultivation ponds each year (Grossman 2011).

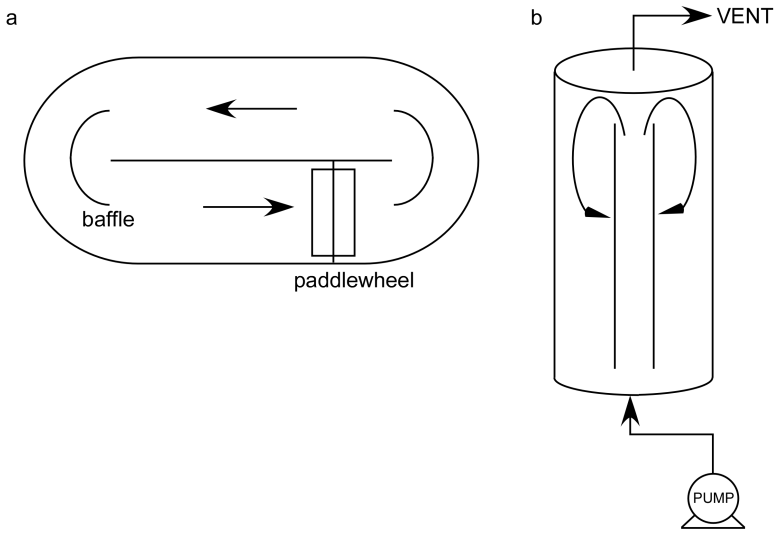


Figure 2.6: Simplified diagram of large-scale algal cultivators: raceway pond (a), photobioreactor (b).

2.5.1.2. Photobioreactors

Growth and oil accumulation can be improved in enclosed cultivation tanks, or photobioreactors (Vasudevan and Briggs 2008). Because the environment can be controlled and is less susceptible to contamination, photobioreactors are often used in biofuel research. A simple photobioreactor design is shown in Figure 2.6b. Arrays of tubular photobioreactors have been popular for production scale-up since the increased surface area to volume ratio allows adequate light penetration into the culture (Chisti 2008). Light capture can be maximized by orientation of the array, and further improved by placement of reflective surfaces. For a given volume, photobioreactors have 13-fold greater productivity than a raceway pond (Chisti 2007).

Cultivation in photobioreactors is much more costly than in raceway ponds. Carbon dioxide and nutrients must be actively supplemented, and more energy expended to circulate the culture and prevent sedimentation. Photobioreactors must be periodically purged of dissolved oxygen and cleaned, and since the culture cannot be cooled by evaporation, temperature must be controlled (Chisti 2007). Because of these added energy inputs, photobioreactors compare unfavorably to lab crops in terms of environmental impact and resource consumption (Clarens et al. 2010). In order to maintain the benefits of closed cultivation, research focuses on decreasing photobioreactor design costs and increasing mass and light transfer efficiency (Lehr and Posten 2009).

2.5.2. Biomass harvest

Once cultivated, the algae biomass must be separated from its growth media so that the media can be returned to the culture for reuse. In this respect, algal biomass

production is more complicated than that of land crop feedstock. Because biomass density is limited by light perfusion, growth is restricted to dilute concentrations. Large growth volumes must be processed in order to recover a substantial amount of biomass (Li et al. 2008). Biomass concentration has been projected at 1-5 g/L (Li et al. 2008), though examples of actual commercial processes usually find culture density to be less than 0.5 g/L (Grima et al. 2003). Since it is so dilute, algae must be concentrated 50 to 200-fold (Grima et al. 2003). A large-scale process would normally require several separation steps, and no single method may be successful for all production modes (Grima et al. 2003).

2.5.2.1. Filtration and settling

Filtration and tank settling are simple, low cost ways to recover algae. Current commercial production modes, such as those for nutraceuticals, use progressive stages of settling ponds and filters in order to remove 20% of water (Spolaore et al. 2006). Gravity or tank settling is used for recovering low-value biomass from wastewater (Grima et al. 2003). Unfortunately, filtration and settling are not ideal for rapid, high throughput processing of large growth volumes. Since microalgae are on scale with bacteria, a filter mesh would need to be so fine as to impede practical flow rates, and settling such small particles would require a long time (Uduman et al. 2010).

2.5.2.2. Centrifugation

Centrifugation is the most reliable and widely used harvest method (Olaizola 2003). It is also expensive, comprising 30% of processing costs (Salim et al. 2010). Hundred-fold concentration requires approximately one kW-h of energy per liter of water (Grima et al. 2003); note that recovering one barrel of crude oil from a raceway pond at 0.2 g/L density and 30% oil content would require centrifuging nearly 3000 L of media.

Despite the high cost, industrial centrifuges can process large volumes of material and yield a highly compact concentrate, simplifying downstream algal processing.

2.5.2.3. Flocculation

Addition of a flocculant induces formation of algal aggregates in suspension (Uduman et al. 2010). It can be used as a preparative step for filtration, gravity sedimentation, or centrifugation by increasing the effective size of the algal particles (Grima et al. 2003). Flocculation is an attractive harvest method because of its low operating cost, but may require long operating times and possibly create reactive compounds (Li et al. 2008). While flocculation has been often employed in wastewater treatment in order to remove unwanted organics, a nontoxic flocculant must be selected for algal harvest so that the growth media can be reused for further cultivation (Uduman et al. 2010).

There are several approaches to algal flocculation. A shift to strongly basic pH 11.8-12 induces flocculation of algal cells due to precipitation of calcium and magnesium hydroxide from the media, which traps algae in salt aggregates (Knuckey et al. 2006). While pH-induced flocculation can be reversed by neutralization of pH, it was found that significant flocculant still remained and the media must be refrigerated before reuse in culture (Knuckey et al. 2006). Efficacy of pH-induced flocculation is dependent on initial algal density and the presence of other ions in the media.

Multivalent cations reduce the negative surface repulsion among anionic polysaccharides of the algal surfaces (Chen and Stewart 2002). Salts such as ferric chloride ($FeCl_3$), aluminum sulfate ($Al_2(SO_4)_3$), and ferric sulfate ($Fe_2(SO_4)_3$) are commonly used (Grima et al. 2003), and their efficacy has been well-demonstrated in wastewater treatment. The flocculation efficiency of metal salts increases with ionic

charge; unfortunately, the presence of metal ions in the biomass may be toxic, and thus is not applicable as animal feedstock (Grima et al. 2003). Also, excess flocculant must be removed before media can be reused for culture (Salim et al. 2010).

Cationic flocculation by polyelectrolytes such as chitosan and polyethyleneimine (PEI) cross-links or bridges algae together (Tilton et al. 1972). Flocculation efficiency is dependent on the polymer molecular weight and dosage, solution pH, ionic charge density, biomass concentration, and extent of media mixing (Grima et al. 2003). Flocculation in seawater is less effective, since the ionic character of seawater causes the polymers to fold tightly and inhibits cationic flocculation; however, the combined use of iron polymer and shift to basic pH has been successfully used to concentrate marine algae (Knuckey et al. 2006).

Anionic materials appear to be ineffective at algal flocculation. Unsuccessful efforts have been described using anionic polymers (Grima et al. 2003) and non-ionic and anionic polyacrylamide (Tilton et al. 1972).

Bioflocculation was demonstrated on *Chlorella vulgaris* and *Neochloris oleoabundans*, using the flocculating algae *Tetraselmis suecica*, *Ankistrodesmus falcatus*, and *Scenedesmus obliquus* (Salim et al. 2010). Flocculation is induced by secretion of biopolymers that cross-link the algae, and no additional chemical flocculant was needed (Salim et al. 2010). The drawback of this method is that a large amount of the flocculating algae could be required in order to flocculate the non-flocculating species; the resulting biomass is then primarily composed of the non-flocculating species, which may not be a high oil-producing species itself.

Ultrasound is a recent separation technique that uses acoustic waves to induce cell aggregation (Bosma et al. 2003). It can concentrate biomass 11-fold at 92% efficiency, but would incur as high of an energy cost as centrifugation. While a novel technique,

ultrasound flocculation would require further development before it could reach commercial application.

2.5.2.4. Drying

Harvested biomass is commonly dried before oil extraction since most extraction solvents are water-immiscible (Raja et al. 2008). In addition, drying prolongs the shelf-life of the biomass, since it can degrade quickly as a concentrate (Grima et al. 2003). After removal of water, the harvested biomass is sun or spray-dried to powder for further processing (Pulz and Gross 2004). Thermal energy input, while decreasing drying time, lessens the sustainability of the process, since drying by oven can require up to 69% of the total energy input (Sander and Murthy 2010) Lyophilization, or freeze drying, is often used in research, but is too expensive for commercial application (Grima et al. 2003).

2.5.3. Oil extraction

After harvesting, oil is extracted from the algae for refinement into fuel products. About 30% of the biomass from large-scale growth could be reasonably assumed to be oil, so throwing away the other 70% of biomass would be impractical. The residual biomass could instead be used for high-protein animal feed, biopolymers, and agricultural fertilizers (Bringezu et al. 2009).

To be recovered, lipids must be either secreted into the extracellular environment of the cell, or extracted by chemical or mechanical means (Chisti and Moo-Young 1986). Unlike crop-based feedstock, oil extraction from algae is unexpectedly difficult. Crops consisting of dry, macroscopic material can be simply pressed in order to obtain oil; unfortunately, high water content of the algal biomass makes it more difficult to press and extract (Banerjee et al. 2002). Since algae are unicellular with high surface area to volume ratio, cell walls are very strong, analogous to a concrete barrier, and resistant to

compressive forces (Chisti and Moo-Young 1986). Efficient oil extraction often requires preparatory drying and mechanical lysis steps, significantly increasing cost (Scott et al. 2010). As a result, most commercial algal harvesting has focused on either secreted products that are easier to recover from the biomass, or high-value products that can be marketed at higher cost.

2.5.3.1. Chemical extraction

The standard method of oil extraction is liquid-liquid separation. The dried algal biomass is treated with hexane, ether, or another organic solvent to draw the oil into solution (Hossain et al. 2008). Scale-up of solvent extraction is difficult, since clumping of the biomass shields itself from further solvent interaction (Banerjee et al. 2002). To improve extraction, the biomass should be continuously mixed within the reactor, especially if residual water is present in the biomass. The solvent is then recovered and distilled, yielding the crude oil extract. At the laboratory scale, specialized equipment such as a Soxhlet apparatus and an accelerated solvent extractor have been developed for improved extraction efficiency, but are intended for smaller dried biomass volumes (milligram scale) and are not scalable for mass processing.

An excess of solvent is used in order to fully saturate the biomass, and since solvents are flammable, safety is a concern. For example, disposal of hexane used for DHA extraction lead to an explosion at a wastewater treatment facility (Feeley 2003). In addition, while the oil extract is considered essentially solvent-free after distillation, toxicity can be an issue for oil products intended for human and animal consumption.

2.5.3.2. Supercritical fluid extraction

Carbon dioxide, with low viscosity and surface tension values, is the most common solvent used for supercritical fluid extraction (SFE) (Letisse et al. 2006). Above

a temperature of 31 °C and pressure of 74 bar, it acts as both a gas and a liquid, and is able to both effuse through and dissolve a material. Carbon dioxide is nontoxic and easily removed from the extract by reducing the pressure. Low operating temperature preserves lipid structure, especially vital for PUFA extraction. However, SFE is about 50% as efficient as industrial hexane extraction (Letisse et al. 2006), and pressurized chambers and pumping equipment are expensive.

2.5.3.3. Continuous culture extraction

"Milking" is the continuous oil extraction from live algal cells. It decreases cultivation costs by recovering oil without destruction of algae. Hydrocarbons have been extracted from the *Botryococcus braunii* extracellular material by brief contact with hexane without decrease in cell viability (Frenz et al. 1989). Furthermore, immobilization of slow-growing *Botryococcus braunii* in gels, gauze, or polymers allowed for continuous extraction of extracellular products without disrupting the culture (Banerjee et al. 2002). *Beta*-carotene has been extracted from *Dunaliella salina* using aqueous dodecane solution, while simultaneously triggering further carotenoid synthesis (Hejazi et al. 2004; Hejazi and Wjiffels 2004). However, other studies found that low oil yields are recovered when milking most algal species, and that further solvent processing is required in order to complete extraction (Sawayama et al. 1995). Currently, culture extraction is at a research phase, and has been most effective with thin-walled or secreting algal species.

2.5.3.4. Solventless extraction

Solid phase extraction is a sorption phenomena in which substrate is coated with the sorbent material and introduced into the biomass in order to selectively bind oil (Nerin et al. 2009). The adsorbed oil can then be recovered from the sorbent by an eluent.

While solid phase extraction is well characterized for analytical applications, its use for algal oil recovery has not been reported on the commercial scale.

Other solventless extraction methods involve liquid-liquid extraction through a partition that prevents cross-contamination of the phases. An extractor using heptane-filled permeable fibers has been used to extract oil of out algal biomass flow-through (Beal et al. 2011). In macro porous polymer extraction (MPPE), hydrocarbons were recovered from water using resin-immobilized solvents (Meer and Brooks 1996).

2.5.3.5. Cell lysis

Extraction efficiency can be improved by preparatory lysis of the biomass. During lysis, the cell wall and membrane of the alga is permanently disrupted by either chemical or mechanical means. Solvent extraction generally acts to both lyse the algae and extract the oil, though combining solvent extraction with a mechanical lysis method improves oil extraction by decreasing the solvent to wet volume ratio required (Lee et al. 1998). Mechanical disruption is achieved by either solid shear methods with grinding apparatuses such as by ball mills or presses, or liquid shear methods with ultrasonics or pressure homogenizers (Chisti and Moo-Young 1986). Application of pulsed electrical fields has also been employed for lysing large volume suspensions of algae (Beal et al. 2011).

2.5.4. Biodiesel synthesis

2.5.4.1. Properties of biodiesel

Biodiesel is a nontoxic, biodegradable fuel composed of long chain-length carbons (C_{12}-C_{24}) (Hossain et al. 2008). In comparison, gasoline is composed of short chain-length hydrocarbons (C_5-C_{12}), while jet fuel is medium chain-length (C_{10}-C_{14}). Biodiesel has a higher energy density than most alternative fuels, making it comparable

to some fossil fuels. Since the energy comes from carbon-fixation, unlike fossil fuels it has a near-neutral carbon footprint (Durrett et al. 2008). Biodiesel is safer than gasoline since it is less volatile; it also has better lubricating properties and a lower emission profile due to its oxygen content (Durrett et al. 2008) (Vasudevan and Briggs 2008) (Gerpen 2005). The physical properties such as cetane number[4] and flash point are comparable to petroleum-based diesel, and biodiesel's low ash content is indicative of a cleaner burning fuel (Vijayaraghavan and Hemanathan 2009). Standard specifications for neat biodiesel (B100) are given in ASTM 6751-02 (Gerpen et al. 2004).

Biodiesel is a transesterified form of triacylglycerol. Being too viscous for direct combustion in diesel engines, the direct use of triacylglycerol would result in poor fuel injection and higher deposit formation (Durrett et al. 2008). As shown in Figure 2.7, transesterification is an acid or base-catalyzed reaction that cleaves the fatty acid groups of triacylglycerol from the glycerol backbone, and uses alcohol to form volatile ester derivatives. Since methanol is commonly used for transesterification, the resulting products are fatty acid methyl esters (FAME).

The fatty acyl composition of FAME influences the resulting biodiesel's physical properties. The bonds in fossil fuel hydrocarbons are mainly saturated, while plant oil acyl chains contain a significant portion of mono- and polyunsaturated bonds. High levels of unsaturated fatty acids leads to better flow properties but a tendency to oxidize, and increased nitrogen oxide emissions (Durrett et al. 2008). The iodine value is used to characterize the extent of unsaturation and oxidative stability (Vasudevan and Briggs 2008).

[4] Cetane number is a measure of ignition delay, combustion duration, engine knocking, and nitrogen oxide emission. Highly branched and aromatic hydrocarbons have lower cetane numbers, and are thus less desirable as fuels.

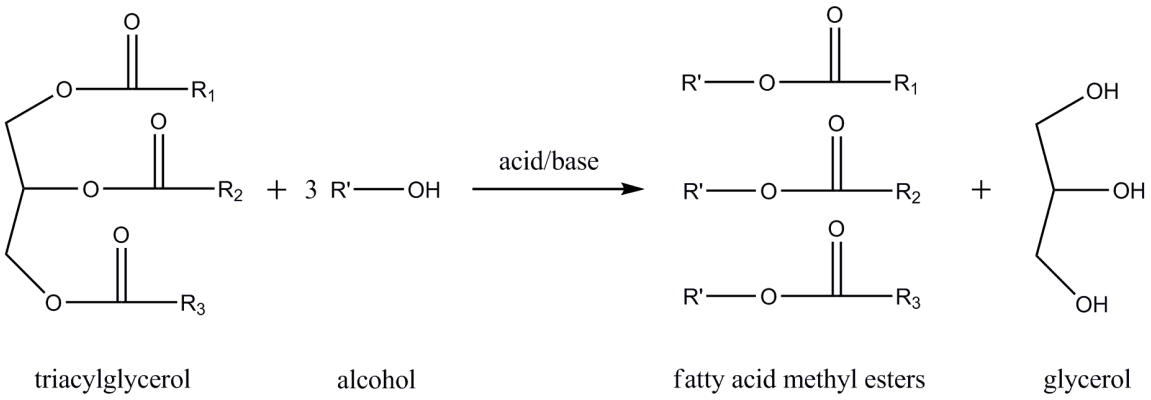


Figure 2.7: Transesterification of triacylglycerol by alcohol yields fatty acid methyl esters and glycerol.

2.5.4.2. Commercial processing

Transesterification is a common industrial process with established alcohol content, catalyst, reaction time, and temperature parameters. Reaction completion is dependent on the ratio of alcohol to oil (Gerpen 2005). While reaction stoichiometry prescribes three moles of alcohol per mole of triacylglycerol to yield three moles of esters, six moles of alcohol are typically used in order to drive the reaction to completion (Chisti 2007). Methanol is the most commonly used alcohol, due to its low cost and ease of recovery, though the use of ethanol has also been reported (Gerpen 2002).

Alkali-catalyzed transesterification is often used for shorter process time (Vasudevan and Briggs 2008), reacting about 4000 times faster than acid-catalyzed reactions (Chisti 2007). Sodium and potassium hydroxide, in 1% concentration by oil weight, are typically used (Chisti 2007). However, base-catalyzed reactions encourage side reactions between free fatty acids and water, leading to soap formation (Vasudevan and Briggs 2008). Soaps are undesirable, as they bind the catalyst in an unusable form

(Gerpen et al. 2004). Soap formation can be avoided by an initial free fatty acid esterification by an acid catalyst, followed by triacylglycerol esterification by alkali catalyst (Serio et al. 2008). Acids such as sulfuric and phosphoric acid can also be used as the sole catalyst, though they generally work slower and require higher alcohol to oil ratios (Gerpen et al. 2004).

Reaction kinetics are temperature-dependent (Gerpen 2005). Transesterification takes about 90 minutes at 60 ºC at atmospheric pressure, with the added benefit of methanol removal by evaporation (Chisti 2007). Reduction to room temperature (about 32 ºC), increases reaction time to four hours (Gerpen 2005). At high reaction temperature extra care must be taken to ensure water-free conditions, since triacylglycerol is susceptible to hydrolysis into diacylglycerol and free fatty acid; water-free conditions can be difficult due to the hygroscopic nature of alcohols (Gerpen et al. 2004).

Approximately ten pounds of glycerol co-product is generated for every 100 pounds of oil transesterified (Gerpen et al. 2004). The water-soluble glycerol separates into the aqueous phase of the reaction product, while FAME, being hydrophobic, is recovered as an oil layer on top. The glycerol is removed by settling or centrifugation, and the methanol distilled for reuse. Glycerol is currently used as an additive for household and personal care products. As the amount of glycerol produced increases concurrently with biodiesel production, it will become cheap enough to use as a biofuel feedstock itself, such as for biohydrogen production or conversion into butyl ethers (Huber et al. 2006), or a substrate for heterotrophic growth (Pyle et al. 2008).

Biocatalysis has been demonstrated in biodiesel synthesis, most notably by microbial lipases acting on vegetable oils (Vasudevan and Briggs 2008). Solid heterogeneous catalysts such as zirconium oxide or zeolite have been investigated in order to solve problems regarding glycerol separation and soap formation (Vasudevan

and Briggs 2008). Increased temperature and pressure conditions have been used to transesterify under supercritical conditions without a catalyst (Gerpen et al. 2004). These novel approaches are still relatively expensive, and have been limited to pretreatment steps.

2.6. THE FUTURE OF COMMERCIAL ALGAE BIOFUEL

Algae are a renewable and sustainable energy source requiring a dedicated research effort in order to realize its potential. Despite the challenges presented, Rodolfi et al. (2009) note that, "given that the transition from fossil to renewable fuels is unavoidable and imminent, and can not rely exclusively on terrestrial plants, any effort to overcome the above limitations and bring microalgae-based fuel production and CO_2 abatement technologies to industrial application is worthy."

Chapter 3: Analysis of Algal Lipids

3.1. Introduction

The identification and measurement of algal lipids has been important to aquatic ecology studies, since algae form the basis of both freshwater and marine ecosystems. More recently, algae have been cultivated for natural products and renewable fuel, and so analytical methods are needed for screening algal lipid content. As there are no set extraction and detection methods for all lipid types, this has lead to varied claims of oil content for different algal species based on the extraction and detection method employed. In addition, lipids encompass a broad range of chemical species, not all of which are equally suitable for nutraceutical or fuel applications. In this chapter, methods for the extraction, separation, and analysis of algal lipids are reviewed.

3.2. Lipid extraction

Because lipids differ from other biological molecules mainly in their hydrophobic nature, the most straightforward manner to separate them is by recovery into an organic solvent. While sample preparation requires extraction of total lipids from residual biomass, methods vary in chemical selectivity, and may not reflect the absolute lipid content of the sample. The physical state of the biomass is important, as residual water in the biomass may inhibit extraction from nonpolar solvents, while a completely dried biomass may effectively shield its interior from complete solvation (Vasudevan and Briggs 2008). Also, solvent interactions may alter the chemical structure of the lipids themselves and produce artifacts. For example, the chemical reaction to derivatize glycerolipids into fatty acid methyl esters may modify the structure of the fatty acids by inducing positional or geometric isomerization (Carrasco-Pancorbo et al. 2009).

Solvent-based extraction methods are largely founded on the procedure developed by Folch et al. (1956), in which the sample is homogenized in chloroform:methanol, and partitioned into organic and aqueous phases by the addition of water. This method has been well-suited to animal tissue studies (Bligh and Dyer 1959; Sheppard 1963; Kates 1964; Hara and Radin 1979). The Bligh and Dyer (1959) extraction is a widely-used modification of the Folch method, and employs a much lower solvent-to-sample ratio, 4:1 compared to 20:1 (Bligh and Dyer 1959). However, the lower solvent ratio is not as effective for extracting material with high lipid content, and it has been pointed out that studies that report using Bligh and Dyer may not be using the published solvent ratios (Iverson et al. 2001).

Besides chloroform:methanol, other solvents have been explored, since the polarity of the solvent affects what types of lipids are recovered. For example, nonpolar solvent systems such as hexane:isopropanol are specific to neutral lipids and largely exclude phospholipids (Guckert et al. 1988), while more polar solvents such as dimethylformamide and acetone have greater selectivity for chlorophyll and other pigments (Wiltshire et al. 2000).

Modifications of the Folch or Bligh and Dyer methods are commonly used for total oil extraction of algae. Since algae have cell walls that are particularly resistant to lysis (Chisti and Moo-Young 1986), extracts have often been prepared with mechanical disruption, which has been shown to improve lipid yields. This has included solvent extraction combined with homogenization (Graeve and Janssen 2009), agitation (Cartens et al. 1996), bead-beater (Lee et al. 1998), grinding (Rai et al. 1997), sonication (Napolitano 1994), and with a Soxhlet apparatus (Guckert et al. 1988).

Recent studies have compared various methods for the purposes of simplifying high-throughput extraction of lipids from algae, and utilizing less toxic solvent systems.

For the most part, modifications of chloroform:methanol extraction methods are still the most effective for frozen or dried biomass, as demonstrated when compared to dichloromethane:methanol (Kumari et al. 2011), aqueous or isopropanol homogenization (Wang and Wang 2011), *tert*-butyl methyl ether or hexane-based solvent systems (Sheng et al. 2011), and Soxhlet extraction with hexane (Ranjan et al. 2010). Studies for scaling-up the extraction of lipids have investigated methods compatible with wet biomass, since traditional nonpolar solvents are not suitable. In some cases, hot alcohol was added to sonicated algal paste so that oil could be recovered after centrifugation (Wang and Wang 2011). In other studies, lipid was extracted from a large quantitiy of wet paste using the Bligh and Dyer method while stirring (Xu et al. 2011). In fact, it has been suggested that algae with weak cell walls can be homogenized without solvent so that lipids would form an emulsion in the aqueous phase, which could then be extracted with hot oil (Cooney et al. 2009). While an attractive option, it would be economically unfeasible given the low concentration of lipid relative to hot oil needed. In our own studies with the high-lipid alga *Chlorella sp*. (Jones et al. 2011), we found that extraction with 2-ethoxyethanol was particularly effective when compared to both chloroform:methanol (Figure 3.1a) and hexane (3.1b). Unlike hexane, 2-ethoxyethanol could be used on wet biomass, and is also less toxic than chloroform.

Direct transesterification has also been useful for analyzing wet biomass since it combines lipid extraction, derivatization, and GC preparation. While water inhibits transesterification, combined use of both acid and base catalyst tolerated up to 10% water content (Griffiths et al. 2010). *Nannochloropsis sp*. and *Chlorella prototheocoides* have been successfully tranesterified with hydrochloric acid, sulfuric acid, or acetyl chloride in methanol, and 1-ethyl-3-methyl imidazolium methyl sulfate (Cooney et al. 2009). Conversion of wet biomass has been demonstrated in catalyst-free conditions, using

subcritical water to hydrolyze the lipids and supercritical ethanol for transesterification (Levine et al. 2010). Moreover, comparison of yields found that direct transesterification of algal biomass generated more FAME than transesterification of extracted material, though direct conversion of wet biomass resulted in 20% less yield than dried biomass (Johnson and Wen 2009).

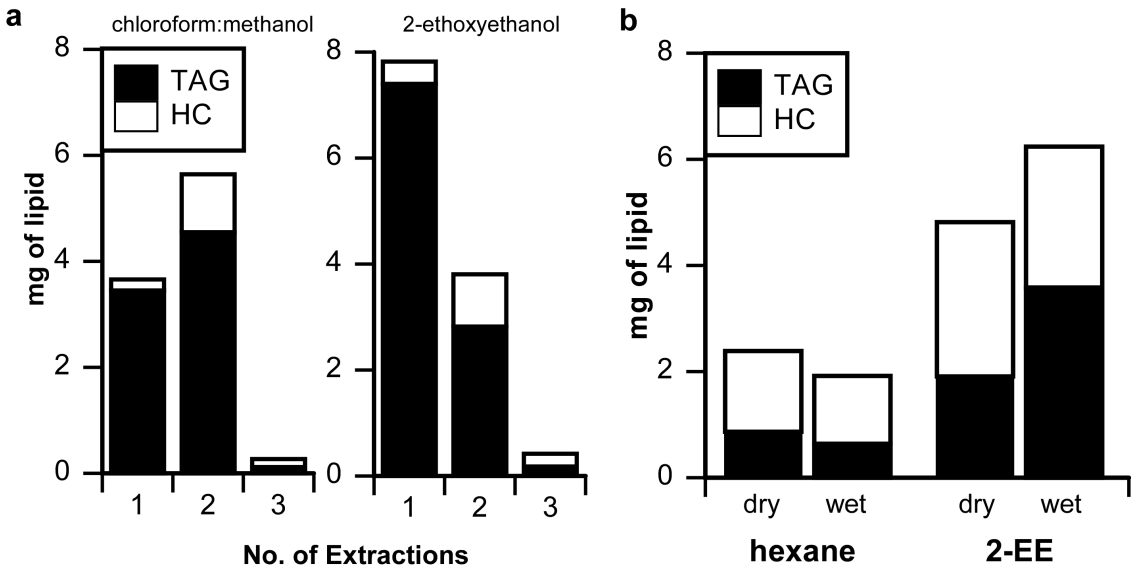


Figure 3.1: Choice of extraction solvent impacts total lipid yield.

Comparison of lipid yields between chloroform:methanol and 2-ethoxyethanol over three consecutive extractions from *Chlorella* sp. (a). Comparison of lipid yields between hexane and 2-ethoxyethanol for dried and wet biomass (b). Quantification based on HPLC-ELSD chromatograms calibrated with lipid standards.

Specialized extraction instrumentation has been developed that use high temperature and high-pressure conditions in order to improve extraction efficiency. A Soxhlet extractor is an apparatus developed for continuous solvent extraction of a sample. It is a distillation-based process in which solvent is refluxed so that it passes through the sample, extracting lipid back into the solvent reservoir. Soxhlet extraction has been routinely used for efficient solvent use and high algal lipid yield from dried biomass (Gao et al. 2008; Li et al. 2008; Sheng et al. 2011). The Soxhlet apparatus is intended for small-scale extraction, so is not applicable to commercial processes.

Accelerated solvent extraction (ASE) applies solvent at high pressure and temperature above its boiling point (Cooney et al. 2009). Extraction of a dried alga and manure mixture with different solvents determined that chloroform:methanol was the best for ASE, and that four cycles were required to extract a comparable yield (Mulbry et al. 2009). Though dried biomass has been extracted with high efficacy, it requires specialized equipment and would be expensive to translate to the commercial scale.

Supercritical fluid extraction (SCF) operates under higher temperature and pressure conditions than ASE, so that the extraction solvent has both gas and liquid properties and effuses through the material. After extraction, the solvent is easily separated and can be reused. SCF using carbon dioxide yielded comparable lipid as Bligh and Dyer extraction (Soh and Zimmerman 2011). The addition of an ethanol entrainer[5] significantly improved yields of SCF-CO_2 extracts (Sun 2005; Machmudah et al. 2006). SCF is ideal since the solvent is nontoxic and selective for neutral lipids, though like ASE the procedure requires specialized equipment and preparatory sample drying and homogenization.

[5] A small quantity of polar cosolvent added in order to improve the solubility of polar analytes.

3.3. CHROMATOGRAPHY

The recovered extract must be fractionated and purified in order to probe the species of interest. Chromatography is based on analyte interactions with a stationary and a mobile phase. The methods include lower resolution, preparative techniques such as thin layer chromatography (TLC) and column chromatography, and higher resolution, instrumentation-based methods such as gas chromatography (GC), and high performance liquid chromatography (HPLC).

3.3.1. Thin layer chromatography

TLC is suited for both lipid species identification and sample fractionation. A silica layer backed with either an aluminum sheet or glass plate serves as the stationary phase, and is eluted with solvent mobile phase. Lipid species are rapidly identified based on comparison with the relative retention times of known standards. Neutral lipids are separated from polar species by chloroform:methanol:water or chloroform:methanol:ammonium hydroxide mobile phase (Rezanka et al. 1988). Species of neutral lipid are resolved by hexane:diethyl ether:acetic acid (Alonso et al. 1998). Once the solvent has been eluted, the analyte spot positions are visualized by iodine stain of unsaturated species, application of sulfuric acid followed by charring, or fluorescent indicator such as Rhodamine 6G (Rezanka et al. 1988). Glycolipids are visualized by sugar stain such as orcinol (Manzi 2001), and glycerophospholipids are visualized by molybdate stain (Skipski et al. 1962). Once resolved, analytes can be scraped from the plate and separated from the silica for further analysis. Figure 3.2 shows an example of a TLC plate of *Neochloris oleoabundans* total oil extract and FAME derivatives (biodiesel)

that was developed with hexane:diethyl ether:acetic acid (90:10:1, v/v/v) in order to resolve neutral lipids and visualized with iodine vapor.

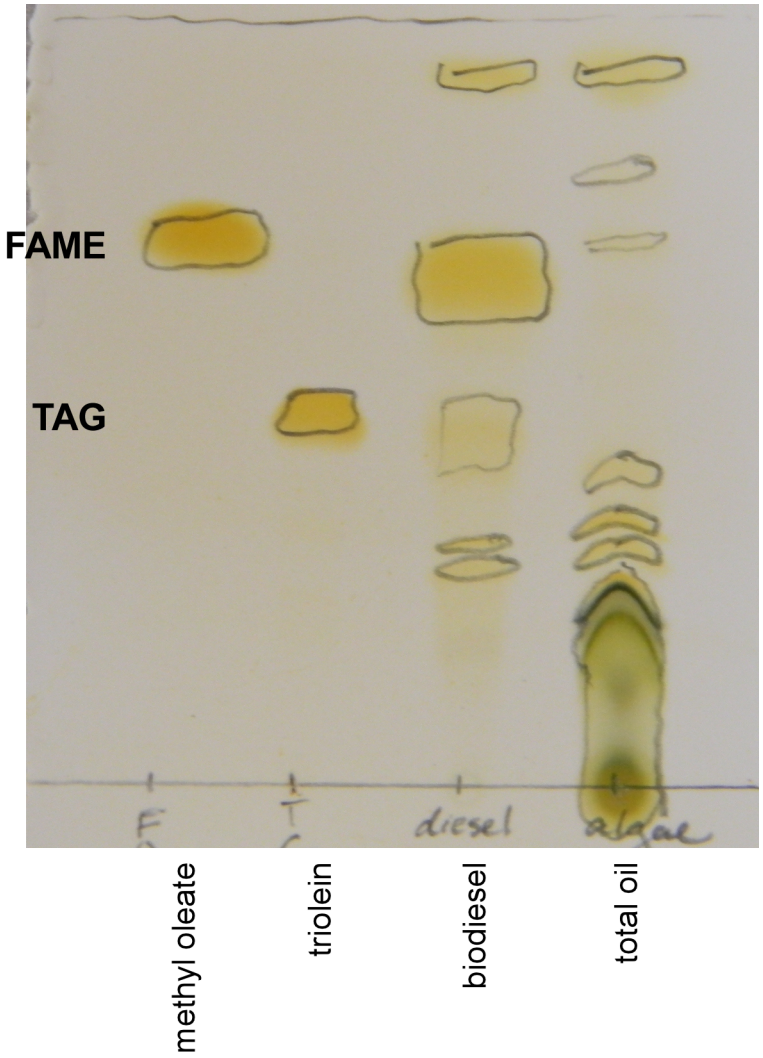


Figure 3.2: TLC of *Neochloris oleoabundans* total oil extract and FAME derivative (biodiesel).

Plate was developed with hexane:diethyl ether:acetic acid (90:10:1, v/v/v) and visualized with iodine. Spots were compared with methyl oleate and triolein standards.

3.3.2. Column chromatography

Column chromatography is a preparative technique that is suited for large sample quantities. Similar to TLC, analytes are eluted by solvent through a solid stationary phase, though in this case a packed column is eluted by gravity, instead of using a thin plate eluted by capillary action as in TLC. Total algal lipid extracts have been separated by class on a silicic acid column by serial elution with hexane, benzene, chloroform, acetone, and methanol (Tornabene et al. 1983; Ben-Amotz et al. 1985), While nominally a low resolution technique, column chromatography can also be used to scale-up lipid purification, such as been shown with an argenated silica gel column for the recovery of eicosapentaenoic acid (EPA) from a fatty acid methyl ester (FAME) mixture (Belarbi et al. 2000).

3.3.3. Gas chromatography

GC is commonly used for analysis of lipids that have been derivatized into FAME (Tornabene et al. 1983), and has been the traditional instrumentation method due to its accessibility. In GC, vaporized analytes interact with an inert stationary phase and a carrier gas mobile phase, before detection by flame ionization detector (FID) or mass spectrometer. In FID, ions are generated and exposed to a voltage differential, generating a current that is proportional to the analyte concentration. The mass spectrometer is described in Section 3.5.2. Since FAME species are separated based on volatility, fatty acyl chain length and degree of unsaturation can be determined. The main drawbacks to GC include the preparation time required, and possible isomerization during transesterification (Carrasco-Pancorbo et al. 2009). Figure 3.3 shows a GC spectrum for hydrocarbon isolated from an unknown algal species found in south Texas.

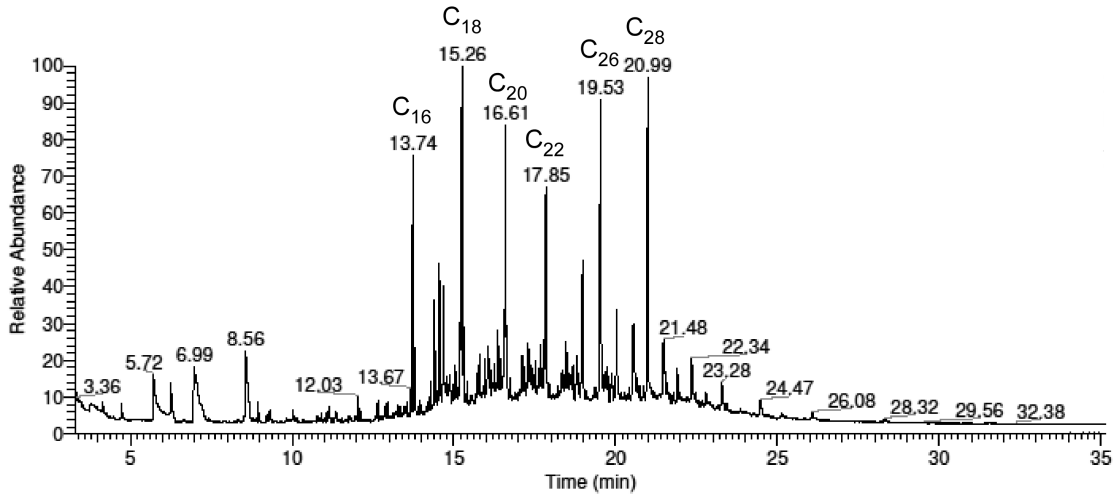


Figure 3.3: GC spectrum of hydrocarbon isolated from south Texas algae.

Isolated hydrocarbon extract from unknown south Texas algal species was composed of linear and branched hydrocarbon chains ranging from C_{16}-C_{28}. Method used silica column with methane gas phase, temperatures 60-320°C.

3.3.4. High performance liquid chromatography

HPLC separates analytes based on polarity, size, or other parameters, similar to TLC and column chromatography. The stationary phase is a column packed with micrometer-sized beads (*e.g.,* silica), providing higher resolution than preparative chromatography, and the mobile phase is liquid solvent. Resolved analytes are detected by evaporative light scattering detector (ELSD), charged aerosol detector (CAD), or mass spectrometer. In ELSD, the analyte is nebulized into light-scattering particles, which produces a detector signal that is proportional to droplet size. In CAD, the analyte is also nebulized before it is passed through a high voltage differential, acquiring an electrical charge. The amount of charge is proportional to the size of the particle. ELSD and CAD

provide comparable detection limits (Ramos et al. 2008), although ELSD signal response is nonlinear at low concentrations. Traditional HPLC detectors based on UV or refractive index are not applicable to lipids due to lack of chromophores and low specificity, respectively. HPLC has become more popular since it is compatible with intact lipids, and with improvements to instrumentation over the past decade.

Two modes are defined for HPLC based on the polarity of the stationary phase. Reversed-phase HPLC uses a column with nonpolar packing and polar mobile phase. It is often used to resolve the individual species within a lipid class based on fatty acyl substituent. Triacylglycerols fractionated from *Phaeodactylum tricornutum* lipid extract were resolved by fatty acyl constituent chain length and position on the glycerol backbone (Yongmanitchai and Ward 1993).

Normal-phase HPLC uses a column packed with polar adsorbent and a nonpolar mobile phase. Both polyvinyl alcohol bonded silica column (Nordback et al. 1998), and monolithic silica column (Graeve and Janssen 2009) have been used to separate marine lipids by class. Xanthophylls, such as astaxanthin, have been characterized using HPLC with chromophore absorbance detection and mass spectrometer (Holtin et al. 2009). As an example, chromatograms of total lipid extract from *Neochloris oleoabundans* and *Chlorella sp*., produced by normal-phase HPLC with a three-gradient solvent system and detected by ELSD (Figure 3.4), show that the *Chlorella* lipid composition is dominated by triacylglycerol, while *Neochloris* contains mainly pigments and phospholipids.

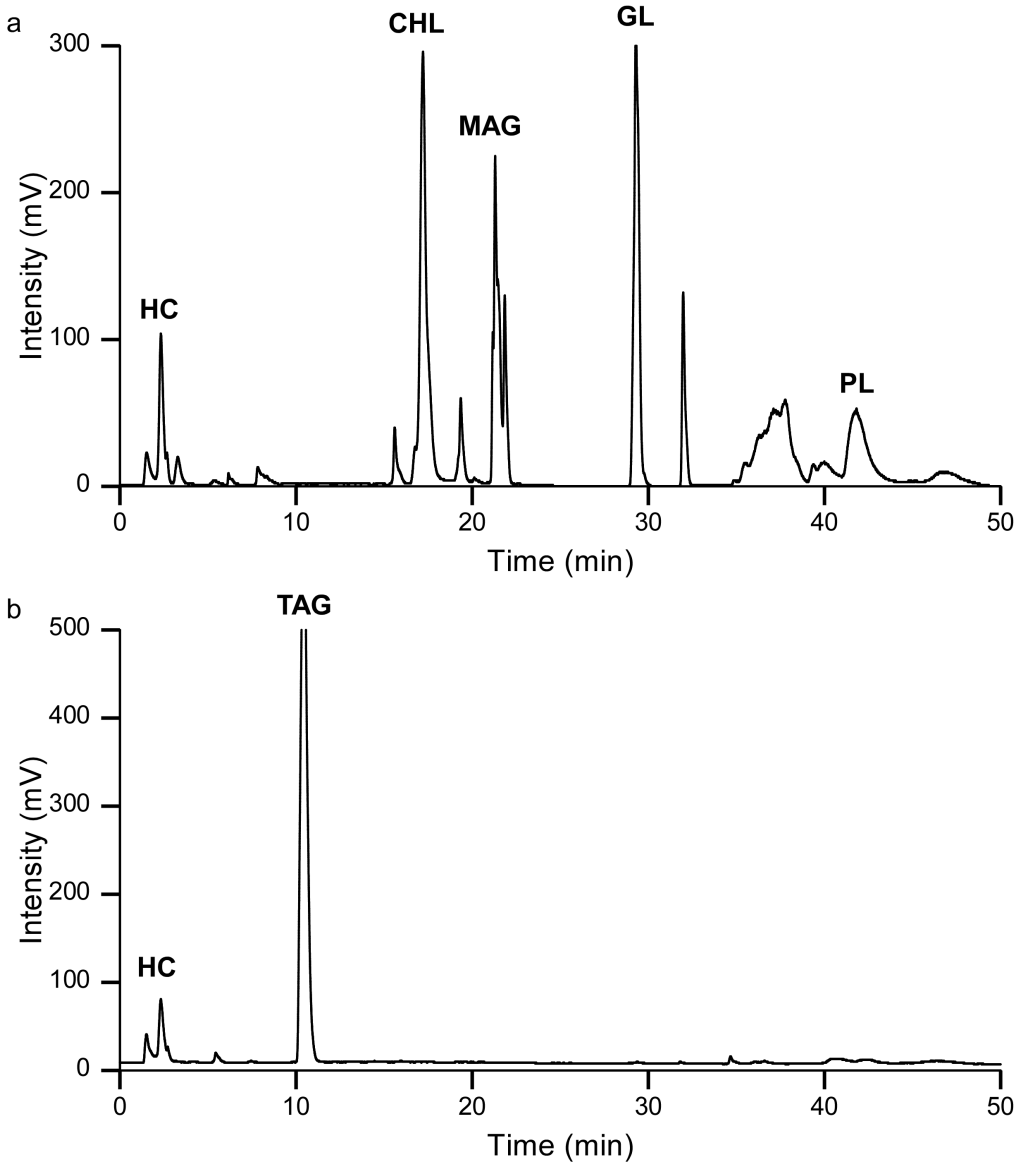


Figure 3.4: Normal-phase HPLC-ELSD chromatograms for two algal species.

Neochloris oleoabundans is composed predominately of chlorophyll (CHL), monoacylglycerol (MAG), glycolipid (GL), and phospholipid (PL) species (a), while lipid profile of Chlorella *sp*. is dominated by hydrocarbon (HC) and triacylglycerol (TAG) lipids (b). Method used polyvinyl alcohol column with isooctane, ethyl acetate, and isopropanol:methanol:water (3:3:1) + 0.1% acetic acid solvent gradient.

3.5. SPECTROSCOPY

Spectroscopy and spectrometry cover a vast array of analytical approaches that detect materials based on interactions with radiated energy fields. Optical spectroscopy, mass spectrometry (MS), nuclear magnetic resonance spectroscopy (NMR), and vibrational spectroscopy methods have been applied for the detection and quantification of lipids in algae.

3.5.1. Optical spectroscopy

The fluorescent activity of lipid probes has been used to detect and quantify algal lipids. A fluorimeter or fluorescence-activated cell sorter (FACS) can be used to obtain quantitative data, and FACS can also screen fluorescence signal from individual alga cells (Alonzo and Mayzaud 1999). They have also been applied to microscopy for imaging of intracellular lipid bodies. Nile Red is a vital dye with blue-shifted emission spectra in hydrophobic environments. Figure 3.5 shows a live suspension of the green alga *Botryococcus braunii* stained with Nile Red and viewed under two emission filters. Cooksey observed increased Nile Red fluorescence with lipid accumulated in nitrogen-deprived algae (Cooksey et al. 1987). Subsequent studies have used Nile Red to distinguish neutral and polar lipids in algae, even those with thick cell walls (Elsey et al. 2007; Liu et al. 2008; Chen et al. 2009). The lipophilic probe BODIPY 505/515 is also useful in that its emission is easily distinguishable from chlorophyll autofluorescence (Cooper et al. 2010). The greatest advantage of fluorescence lipid detection methods is that no solvent extraction is necessary and they can be applied to small sample sizes, allowing for more rapid screening of algae than traditional chromatographic methods.

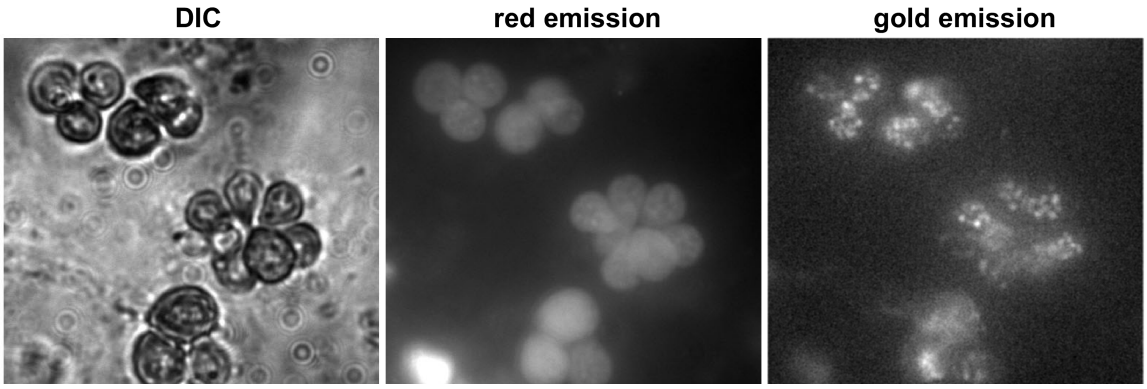


Figure 3.5: Nile Red staining of *Botryoccocus braunii*.

Differential interference contrast (DIC) shows the form and outline of the cell aggregates. Chlorophyll autofluorescence is visible under red emission filters. When viewed with yellow emission filters, the neutral lipids of the oil storage bodies are visible within the cells.

3.5.2. Mass spectrometry

Improvements to MS instrumentation have enabled qualitative and quantitative analysis of individual analytes in a complex lipid mixture. In MS, the analyte is ionized and separated by magnetic and electric field interactions in order to determine its mass, structure, and/or composition. MS can be applied on its own to an extract, or as a detector for GC or HPLC separation systems. After the sample is ionized, mass analyzers sort ions based on mass to charge ratio, and include quadrupole, ion trap, time of flight (TOF), and Fourier-transform ion cyclotron resonance (FT-ICR) instruments. The detectors differ in mass range, mass accuracy, and resolution, and are often associated with particular ionization sources.

Initial advances with MS used hard ionization techniques that fragment an analyte into many charged ions in spectra. Electron ionization (EI) is generated from the impact of highly energetic electrons, and has created reproducible fragmentation patterns from

which public databases have been compiled (Carrasco-Pancorbo et al. 2009). However, EI spectra tend to obscure the molecular ion species, and thus chemical ionization (CI), a lower energy process, has also been used for producing simplified spectra. In CI, an ionized reagent gas, usually methane, ammonia, or isobutane, collides with the analyte in order to produce ions. EI and CI methods have been used for characterization of algal toxins and pigments, and for probing simpler lipids such as FAME from *Schizochytrium sp*. (Michaud et al. 2002). Figure 3.6 shows CI-MS spectra for two types of hydrocarbon found in an unknown south Texas alga.

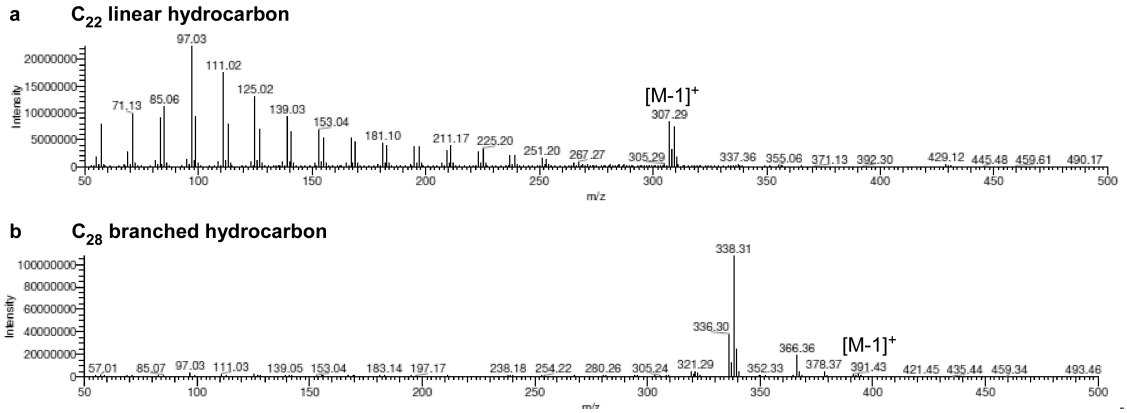


Figure 3.6: CI-MS spectra of hydrocarbons in unknown algal species from south Texas.

CI fragmentation patterns give qualitative information on the conformation of hydrocarbon species. Molecular ion species is characterized by loss of one m/z $[M-1]^+$. Linear hydrocarbons yield regular fragments of similar magnitude of signal intensity with difference of 14 m/z (a), while hydrocarbons with branch points typically are dominated by fragments associated with stable carbocation formation (b).

Electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and matrix-assisted laser desorption ionizaton (MALDI) are soft ionization techniques that result in spectra dominated by molecular ion species. They have been successful for the ionization of biological macromolecules, and are often employed for MS analysis of lipids. ESI and MALDI are appropriate for analytes that can be charged, such as polar lipids, while APCI is better suited for neutral species (Byrdwell 2001).

In ESI, ions are formed when the liquid analyte droplet is exposed to high voltage reaching a critical point that causes it to disperse into small, charged particles. ESI has often been used for the analysis of polar lipids, and tandem MS has enabled clear identification of carbon chain length and degree of unsaturation in a complex mixture (Carrasco-Pancorbo et al. 2009). Sample preparation with ammonium formate or sodium acetate has been shown to improve sensitivity by forming salt adducts that can be predicted and detected (Byrdwell and Neff 2004). ESI has been used for non-phosphorus-containing polar lipid analysis in the chlorarachinophyte class (Roche and Leblond 2010). Coupled to HPLC, it was used to identify long-chain fatty acyl substituents in a polar lipid mixture (Herrero et al. 2007) and in the chloroplast glycolipid fraction of a large set of algal species (Gray et al. 2009).

APCI is particularly useful for neutral lipid analysis since its gentle ionization produces useful diagnostic fragment ions (Carrasco-Pancorbo et al. 2009). APCI is similar to ESI and is often packaged together in current commercial systems. Ions are generated by APCI when nebulized analyte particles interact with high voltage. In studies with *Scenedesmus dimorphus*, HPLC-APCI/MS was used to analyze FAME derivatives and total lipid extracted by SCF-CO_2 (Soh and Zimmerman 2011), and astaxanthin esters in *Haematococcus pluvalis* (Miao et al. 2006; Holtin et al. 2009). Figure 3.4 shows

APCI-MS positive mode spectra for FAME generated from *Neochloris oleoabundans*, before and after nitrogen deprivation. Stressed algae showed an increase in palmitate esters (C16:0) derived from the increased triacylglycerol content of the algae.

MALDI is a rapid MS technique that does not require front-end chromatography. The analyte, embedded in a matrix, is desorbed and ionized by a laser source. While MALDI has been very popular in the field of proteomics, it has also been applied to lipidomics. Lipid classes from *Chlamydomonas reinhardtii* and *Cyclotella meneghiniana* were separated by TLC then analyzed by MALDI-TOF in order to identify acyl substituents of chloroplast glyco-, sulfo-, and phospholipids (Vieler et al. 2007).

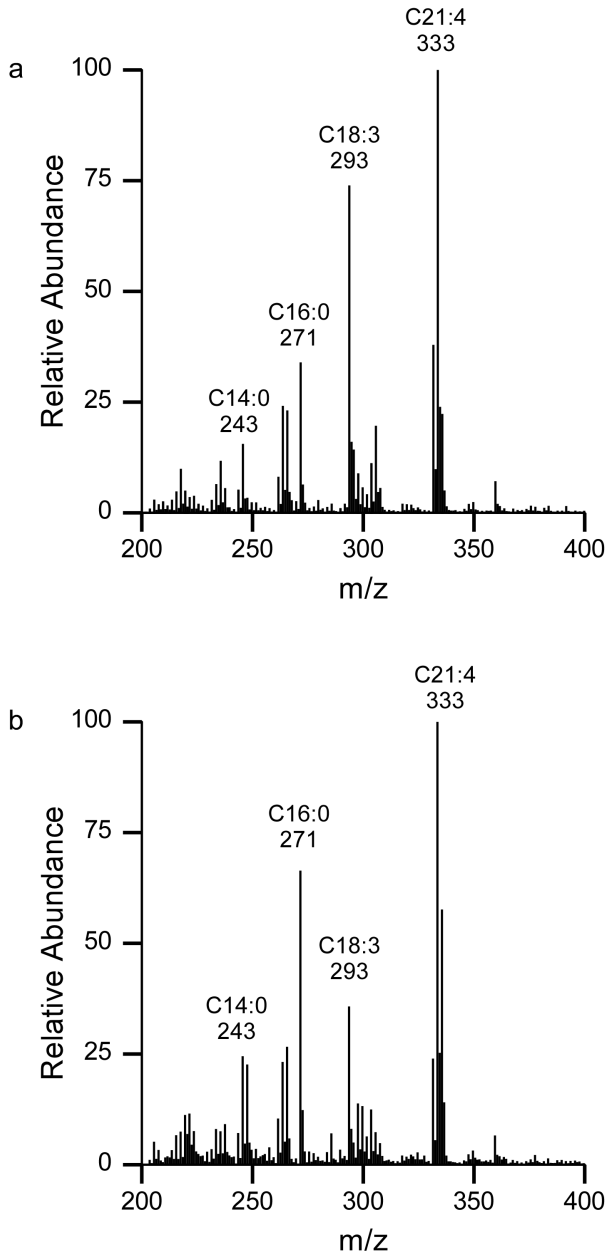


Figure 3.7: APCI-MS positive mode spectra for FAME.

Spectra show difference relative distributions of chain length and unsaturation in FAME. Samples were generated from base and acid-catalyzed transesterification of *Neochloris oleoabundans* cultured in nitrogen-complete media (a) and nitrogen-deficient media (b).

3.5.3. Nuclear magnetic resonance spectroscopy

NMR protocols have been developed for probing both lipid extracts and even whole algal cells. In NMR, atomic nuclei are aligned by a strong uniform magnetic field. Since nuclei relaxation and its subsequent resonant frequency is characteristic of the nuclei, the detectable chemical shifts in the resulting NMR spectrum that can be used to determine lipid structure. ^{1}H and ^{13}C NMR has been used to confirm hydrocarbon species in cell walls of freshwater algae (Allard et al. 2002). ^{1}H NMR has been used to characterize carotenoids in *Haematococcus pluvialis* (Holtin et al. 2009). ^{13}C NMR has been used to identify species in a complex algal extract (Pollesello et al. 1993). Lipid content in *Chlorella protothecoides* was determined by time domain-NMR based on differences in frequency shift between water and oil phases (Gao et al. 2008); accuracy of the quantification was comparable to gravimetric measurement of the Soxhlet extract. The main difficulties with using NMR for lipid analysis are the overlap of signals in ^{1}H NMR, and low natural abundance for ^{13}C NMR (Carrasco-Pancorbo et al. 2009). Figure 3.8 shows the ^{1}H NMR spectrum of hydrocarbon extracted from an unknown species of algae from south Texas.

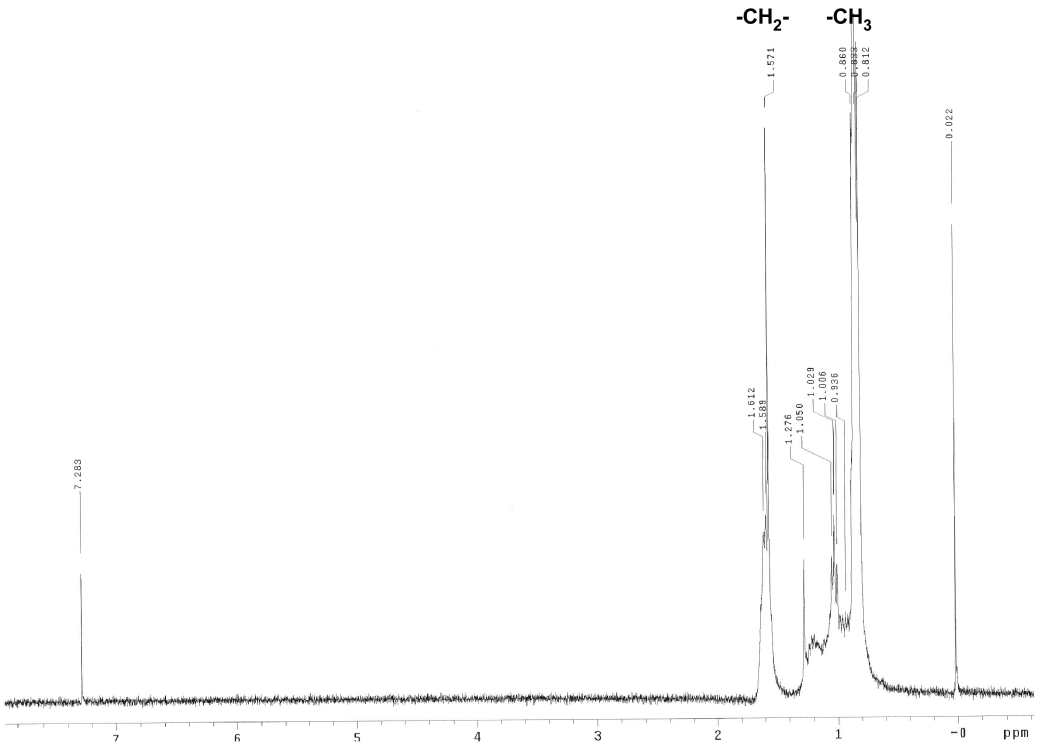


Figure 3.8: ^{1}H NMR spectrum of hydrocarbon extract from unknown algal species from south Texas.

^{1}H NMR spectrum shows that the hydrocarbon fraction was composed of alkanes, as indicated by chemical shifts around 0.5 ppm (methyl) and 1.5 ppm (alkyl). Downstream artifact peak was due to chloroform solvent.

3.5.4. Vibrational spectroscopy

Other non-destructive spectroscopic methods for lipid analysis include IR and Raman, which detect the vibrational energies of molecules. Based on its structure, a molecule can absorb energy at certain frequencies. In infrared spectroscopy (IR), absorption of energy in the infrared spectrum induces vibrational excitation of covalent bonds, which include movements such as bond stretching, bending, scissoring, rocking, and twisting. IR application has improved with the advent of Fourier-transform infrared spectroscopy (FTIR). FTIR was used in detect isolates of algenans in the cell walls of freshwater algae (Allard et al. 2002). IR and FTIR were used to create a high-throughput screening method to distinguish polar and neutral lipids in algal biomass (Laurens and Wolfrum 2011). Like NMR, the main difficulty with IR methods involve signal overlap in a complex lipid mixture, though computing advances will continue to streamline such problems.

Complementary to IR, Raman spectroscopy yields spectra based on the vibrational frequencies of molecules. While IR spectra show the percent of energy that is absorbed at a given IR frequency, Raman signal intensity is due to the inelastic scatter of an excitation source. Specifically, the Raman shift occurs when the excited electron of the molecule relaxes to a different energy state due to molecular vibration. Raman spectroscopy has been used to probe the accumulation of hydrocarbon in *Botryococcus braunii* (Banerjee et al. 2002), and triacylglycerol content in *Chlorella sorokiniana* and *Neochloris oleoabundans* (Huang et al. 2010). In addition to signal overlap problems seen with IR, Raman can be difficult to implement due to typically low signal intensities and signal-to-noise ratios; these problems are being addressed by improved laser sources and detectors.

3.5. CONCLUSION

Lipids are a diverse group of molecules, and no one extraction or analytical method is suited for all types. Screening of lipid content will improve biofuel production economics, and guide genetic manipulation to improve lipid synthesis. Methods to extract lipids from the intracellular environment vary with water-miscibility and lipid class specificity, and must be selected based on the desired application. Recent advancements in analytical instrumentation enable more precise quantification and finer probing of the algal lipidome.

Chapter 4: Oil binding and biomass separation by hydrophobic resins

4.1. Introduction

Renewable biofuels are being developed to address future energy shortages. Algae are optimal feedstock due to their high biomass productivity and lipid content (Chisti 2007). In addition, algae are more sustainable than land crops. Cultivation would not draw from the food supply and non-arable land can be used (Chisti 2008). Unfortunately, oil extraction is more difficult from algae than from terrestrial plants, making algae-based biofuel prohibitively expensive; new technologies are needed in order to achieve sustainable large-scale biofuel production (Scott et al. 2010).

Oil extraction is costly due to two major problems. First, algae grow in dilute suspension, and must be dewatered before processing. Mechanical extraction processes commonly used for land crops, such as pressing, cannot be applied to wet algal biomasses (Cooney et al. 2009). Algal oil is usually extracted with organic solvents, either hexane or ether, that are water-immiscible. This makes drying the algal biomass necessary, but heating is expensive, the cost of which can comprise up to 70% of processing costs (Sander and Murthy 2010). Additionally, it has been found that too much drying can also impede oil extraction. The dried biomass can form a solvent resistant material, in which the exterior surface effectively shields solvent penetration (Vijayaraghavan and Hemanathan 2009).

The other problem with algal oil extraction is that even when a dewatered algal biomass is obtained, the intracellular lipids are difficult to liberate. This is due to the unexpectedly strong protection conferred onto the alga by its cell wall (Chisti and Moo-Young 1986). The cell wall can be lysed by chemical or mechanical means. Solvent application can both lyse cells and extract oil, but sufficiently large volumes must be

applied. Mechanical methods apply shear forces to lyse cells, and require equipment such as homogenizers, ball mills, and sonicators (Chisti and Moo-Young 1986). Even though lysis improves oil extraction, its use must be weighed against the cost it adds to the production process.

Because of these obstacles, current commercial algal processes either harvest secreted products that are easier to recover, or extract oil products that can be sold at a higher cost (Chisti and Moo-Young 1986). Neither of these recourses is suitable for biodiesel production. Biodiesel is generated mainly from triacylglycerol, which is found in intracellular storage compartments that are not easily accessible. In addition, biodiesel must compete with petroleum fuel, a low-value product, and so must be made cheaply.

Process economics can be improved by producing chemical feedstock, biopolymers, and agricultural fertilizers from oil-extracted residual biomass (Bringezu et al. 2009). In fact, it has been suggested that reusing the biomass is critical to achieving process efficiency (Xu et al. 2011). Some algal species are already used in human food and health products (Singh et al. 2005). The algal biomass can also be recycled as high-protein animal feed; livestock fed an alga-supplemented diet have improved immune responses and increased productivity (Pulz and Gross 2004).

For products for human, animal, or agricultural use, contamination of the biomass with extraction solvents is an issue. Despite solvent removal by evaporation, there is public concern over residual hexane content in the extracted material used for food products such as docosahexanoic acid (DHA) in infant formula (Letisse et al. 2006). There has also been problems with disposal of extracted biomass; for example, an explosion was reported due to disposal of hexane-contaminated biomass at a wastewater treatment facility (Feeley 2003).

Alternative solvent extraction methods have been explored. Biocompatible solvent extraction processes use ethanol or ethanol mixtures (Medina et al. 1998). While effective, they require further purification of the crude oil as non-lipid components are also extracted. In supercritical fluid (SCF) extraction, a nontoxic, non-flammable compound such as carbon dioxide is used as the solvent by subjecting it to increased temperature and pressure (Medina et al. 1998). SCF extraction has been successfully used for extraction of high-value compounds such as the carotenoid astaxanthin, but its capital and operating costs are prohibitively expensive for biofuels.

Solventless separation methods are a possible alternative approach to oil extraction that does not require complete biomass drying. Solid phase extraction is commonly used for analytical preparation. Instead of partitioning compounds into different liquid phases based on solubility, solid phase extraction involves adsorption onto a substrate (Nerin et al. 2009). Large-scale chromatographic methods, such as with a silicic acid column, have already been applied for separation of triacylglycerols (Medina et al. 1998). Another solventless approach is to separate by liquid extraction, while maintaining a selectively permeable barrier between the two solvent systems. Macroporous polymer extraction (MPPE) uses solvent immobilized to resin to separate hydrocarbons out of aqueous suspension (Meer and Brooks 1996). A hollow fiber membrane extractor, using permeable tubes to separate solvent and aqueous phases, has been used to recover oil from an algal suspension (Beal et al. 2011). By keeping the organic phase separate from the aqueous suspension, the residual biomass can be recovered free from solvent contamination.

We have explored the use of hydrophobic polymers for separating oil and residual algal biomass. Here, lysed algae are passed over a resin surface that accumulates oil out of the biomass as it passes over the resin. The oil bound to the resin can then be eluted

with relatively small volumes of solvent that never contact the biomass. Different resins were synthesized and compared for extraction efficiency and binding capacity.

4.2. MATERIALS AND METHODS

Monomers di(ethylethene glycol) vinyl ether (DEG), dimethylamine methacrylate (DMAEMA), divinylbenzene (DVB), ethylene glycol dimethacrylate (EGDMA), hexyl methacrylate (hexMA), methyl methacrylate (MMA), styrene (S), and 2-hydroxyethyl methacrylate (HEMA), and azobisisobutyronitrile (AIBN) were obtained from Sigma-Aldrich (St. Louis, MO). Corn oil was purchased locally.

Nile Red was obtained from Sigma-Aldrich and prepared as a 10 mM stock solution using anhydrous dimethyl sulfoxide. Sytox Green was obtained from Invitrogen (Carlsbad, CA) as a 5 mM stock solution.

A species of saltwater *Chlorella* (KAS 603) was obtained from Kuehnle Agro Systems (Hawaii).

HPLC solvents ethyl acetate, isooctane, methanol, toluene, and water were of ACS grade, and were degassed and filtered prior to use. HPLC lipid standards included mineral oil (purchased locally), purified chlorophyll b extract from spinach (Sigma-Aldrich), and tripalmitin (Sigma-Aldrich).

4.2.1. Resin synthesis

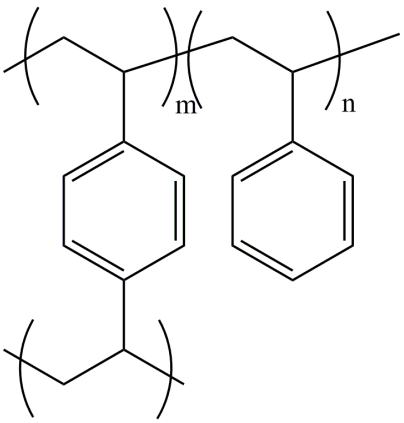
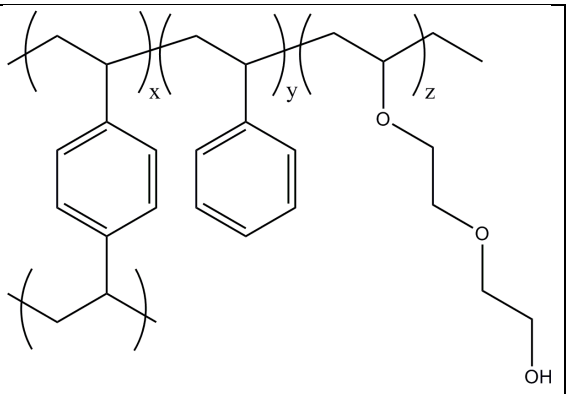
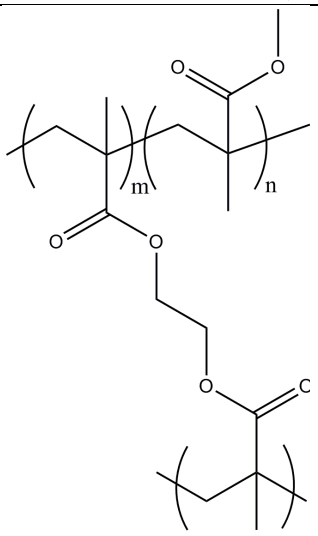
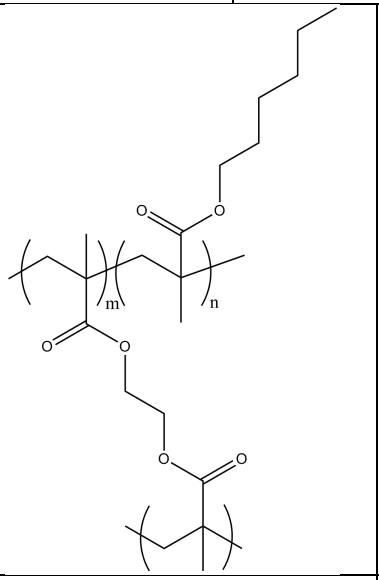
Monomers were combined in ratios based on weight as indicated in Table 4.1. Crosslinkers, EGDMA or DVB, were incorporated at 80% wt in order to form polymethacrylate (PMA) and polystyrene (PS) resins, respectively. Remainder of resin weight was occupied by spacer monomers: MMA for PMA resin (JB20/21), or S for PS

resin (JB8/10). Functional monomers DMAEMA, HEMA, hexMA and DEG were incorporated into resins JB40, JB1-13, and SN03 as indicated.

An equivalent volume of organic solvent (*i.e.*, toluene) was added to the combined monomers. Molecularly imprinted polymers (Beach and Shea 1994) were generated by polymerization in the presence of corn oil to create binding sites specific to triacylglycerol. 1 mol percent AIBN was added and the mixture was heated in 60 °C water bath and continuously stirred under argon until resin polymerized into a brittle solid. The resin was dried in 55 °C oven (1 atm) for 12 h before crushing with mortar and pestle. Corn oil was washed from imprinted polymers using acetone and hexane. The crushed resin was then sized between 35 and 170 size stainless steel meshes to obtain macroporous beads of approximately 100-500 um diameter.

4.2.2. Vegetable oil binding assay

Dried resin (2 g) was weighed into a 10 mL polypropylene column (Pierce). Resin was wetted with distilled water before adding 10 mL mixture of 5% corn oil emulsion (prepared by pulsed sonication of oil and water mixture) stained with 1 mM Nile Red. Unbound oil emulsion was collected from column, then extracted with acetone to draw oil out of water. Resin-bound oil was eluted from resin with acetone. Nile Red optical density was measured for unbound and resin-bound oil using spectrophotometer (Shimadzu) at 550 nm with acetone blank in order to determine the percent of oil retained to the resin.

	
DVB:S (JB8/10) m = 80, n = 20	DVB:S:DEG (SN03) x = 60, y = 30, z = 10
	
EGDMA:MMA (JB20/21) m = 80, n = 20	EGDMA:hexMA (JB1-13A/B) m = 80, n = 20

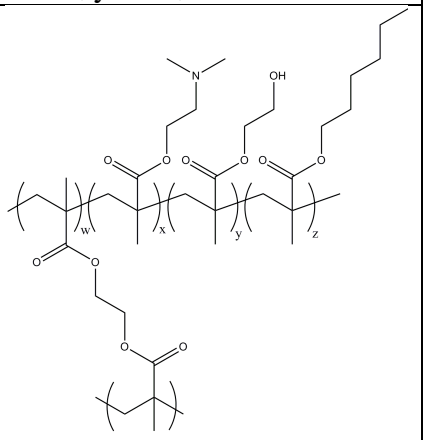

EGDMA:DMAEMA:HEMA: hexMA (JB40) w = 70, x = 17, y = 7, z = 6

Table 4.1: Hydrophobic resin structures.

4.2.3. Algal cultivation and harvest

Chlorella sp. was cultivated in saltwater (approximately 35 psu) f/2 medium (Jeffrey and LeRoi 1997). Culture was grown at room temperature (23 ºC) under cool white fluorescent lights on a 12 h:12 h, light:dark photoperiod in airlift photobioreactor. Algae were harvested after approximately 14 days of growth, and mechanically lysed by electrodistention (Center for Electromechanics, University of Texas at Austin), French press, sonication, freeze-thaw, or used without treatment.

4.2.4. Algal oil accumulation assay

2 g of dried resin was weighed into a 10 mL polypropylene column. Resin was wetted with distilled water before adding 50 mL of algae suspension. Unbound algal suspension was collected from column, along with 50 mL of distilled water used to rinse the column. Resin-bound algal oil was eluted from the column using 50 mL of hexane:acetone (3:1, v/v). Solvent was evaporated from oil elution using rotating evaporation and high pressure pump. Sample was resuspended in 1 mL of toluene for HPLC analysis.

Two sets of 2 mL of algal suspension (0.3 g/L density) and 1 mg corn oil in 2 mL sonicated emulsion were either combined into a mixture by pipeting, or loaded onto separate resin columns containing 2 g of JB20. After loading, the resin-bound oil was recovered by solvent elution as stated above and quantified by HPLC analysis.

4.2.5. Algal biomass oil extraction

Oil was extracted from unbound algal suspension by the Folch method (1956). Suspension was pelleted by centrifugation (5000 RPM, 10 min) and supernatant removed. Pellet was then refluxed in 50 mL of chloroform:methanol (3:1, v/v) for 30

minutes at 60 ºC while stirring. Solvent was separated from the biomass by filtering through a 0.47 μm PTFE membrane (Millipore, Billerica, MA). The biomass and membrane were transferred back into a flask and refluxed as before. The filtered solvents were combined and placed in separatory funnel with 0.2 volume of 0.9% sodium chloride (w/v). After mixing, the extract was allowed to settle into a biphasic solution. The upper organic phase was recovered, and the solvent removed by rotating evaporation and high vacuum pump. Sample was resuspended in 1 mL of toluene for HPLC analysis.

4.2.6. Quantification by HPLC-ELSD

In preliminary studies, resin-bound algal oil and unbound algal oil were quantitatively analyzed for neutral lipid species content by high performance liquid chromatography (HPLC) with evaporative light scattering detector (ELSD) (Sedere Sedex 75). Analog data from the Sedex 75 ELS detector was supplied to the PC through an A/D data acquisition box (Agilent Technologies, SS420X).

Lipid standards and extracts were resolved using a normal-phase silica column (Thermo Scientific Hypersil Silica 250 mm x 4.6 mm I.D., 3 μm bead size). The column was protected by a Waters Guard-Pak™ guard column containing Nova-Pak™ silica inserts. Isocratic HPLC pump (Series III) was run at 0.7 mL/min flow rate for 12 min with mobile phase consisting of toluene + 0.5% methanol. 20 μL of sample were manually injected with Hamilton syringe. Identity of triacylglycerol component was determined by comparison with standard retention time.

4.2.7. Quantification by gradient HPLC-ELSD

During the course of the study, the HPLC system was upgraded to a multi-solvent gradient pump with autosampler (Thermo Finnegan Surveyor). ELSD and A/D data acquisition remained the same as stated in previous section.

Lipid standards and extracts were resolved using a normal-phase poly-vinyl alcohol-bonded silica column (YMC Pack PVA-Sil-NP 250 mm x 4.6 mm I.D., 5 μm bead size) with same column guard as stated previously. The mobile phase consisted of a three-solvent system, with gradient method given in Table 4.2. Lipid class identification was determined as stated in previous section.

time (min)	flow rate (mL/min)	A (%)	B (%)	C (%)
0	1.5	100	0	0
5	1.5	98	2	0
15	1.5	75	25	0
19	1.5	20	80	0
24	1.5	0	100	0
32	1.3	0	50	50
38	1.0	0	15	85
43	1.0	0	0	100
52	1.0	0	100	0
54	1.0	0	100	0
60	1.5	90	10	0
64	1.5	100	0	0
74	1.5	100	0	0

Table 4.2: HPLC mobile phase gradient method.

Three-solvent system of iso-octane (A), ethyl acetate (B), and isopropanol:methanol:water (3:3:1, v/v/v) + 0.1% acetic acid (C) (Jones et al. 2011).

4.2.8. Ethanol treatment and verification of cell membrane disruption

Algal biomass in growth media, approximately 0.3 g/L density, was combined with 0.2 volume of ethanol and stirred for 15 minutes at either room temperature (23°C) or 60 °C reflux. After cooling, the sample was tested for oil retention by the resin.

Algal cell membrane disruption was confirmed by visualizing cells using a inverted fluorescence microscope (Nikon Diaphot 200, Hamamatsu Orca CCD) with 100X objective (Nikon Plan Apo 100X/1.40 oil). 1 mL of algae was incubated at room temperature for 15 min with 1 μM of membrane-impermeable nucleic acid stain Sytox Green. The cells were pelleted by centrifugation and resuspended in 1 mL of growth media. The cells were washed in this manner three times. Approximately 200 μL of cell suspension was placed onto a #1 coverslip and observed immediately by microscope.

4.3. RESULTS

Figure 4.1 illustrates the vegetable oil binding assay (a-e) and algal binding assay (f-i). In the vegetable oil binding assay, polymer were screened by spectrophotometric comparison of the optical density of Nile Red in the resin-bound eluate and aqueous flow-through suspension. Resins were then tested for oil binding from algal suspensions. Oil eluted from the resin and oil remaining in biomass flow-through were quantified by HPLC.

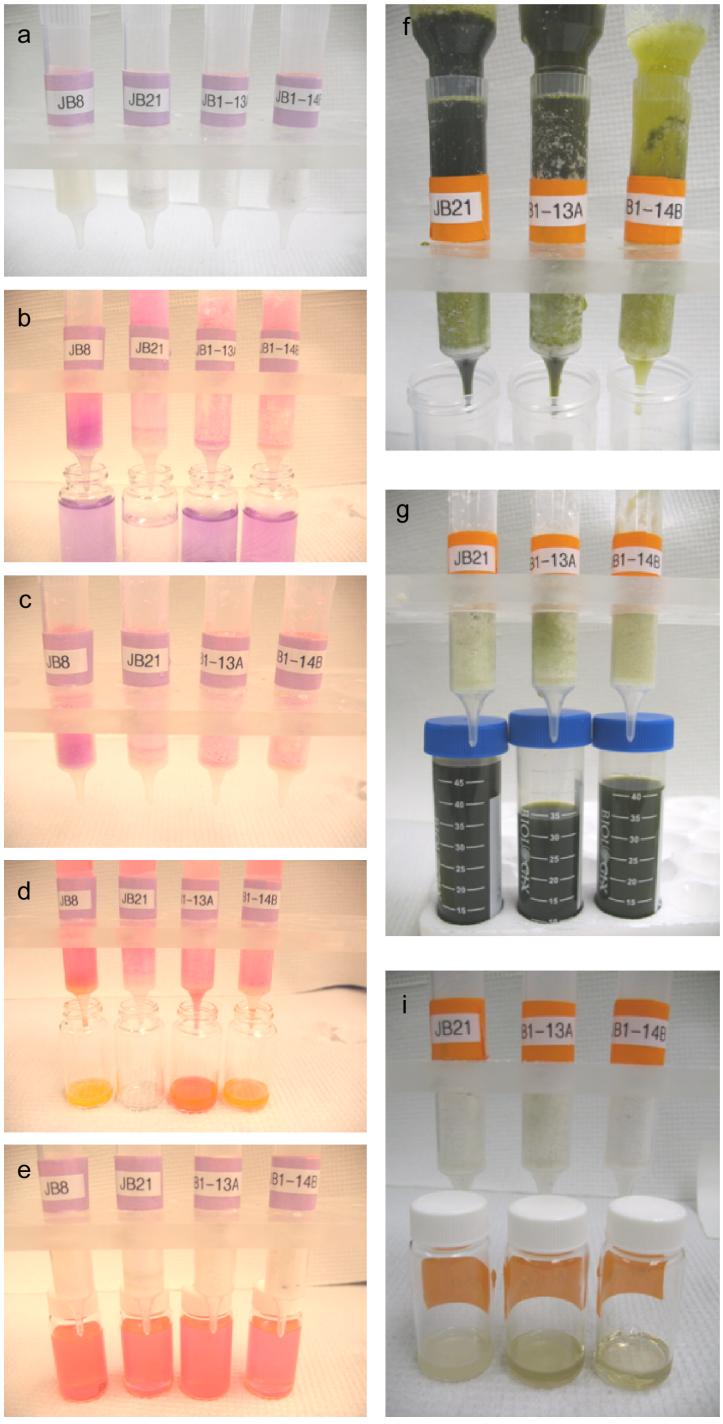


Figure 4.1: Photo illustration of vegetable oil and algal oil binding assays.

Resin were weighed into columns (a). Nile Red-labeled vegetable oil emulsion was loaded onto the resin (b). Oil retained onto the resin colored the columns pinkish-red (c). Elution of column recovered oil from the resin (d). Recovered solvent elutions (e) were compared with unbound oil staining in aqueous emulsion (b) by spectrophotometry to calculate ratio of oil that was retained by the resin. Alternatively, algal suspension was loaded onto the resin (f). Algal oil was retained by the resin, whereas residual biomass was eluted with aqueous suspension (g). Elution of column recovered oil from the resin (i). The oil content of the resin eluate (i) and unbound residual biomass (g) were quantified by HPLC in order to calculate ratio of oil that was retained by the resin.

Resin synthesis protocols were evaluated by screening initial resin products with the vegetable oil binding assay. Figure 4.2 shows PMA (JB20), imprinted PMA (JB21), and PS (JB8) resins retained 85.1%, 84.5%, and 90.2% of oil respectively out of emulsion. Addition of hydrophobic indicator Nile Red allowed for both quantification and visual confirmation of oil retention, since the resin would appear pink/red only when binding the oil.

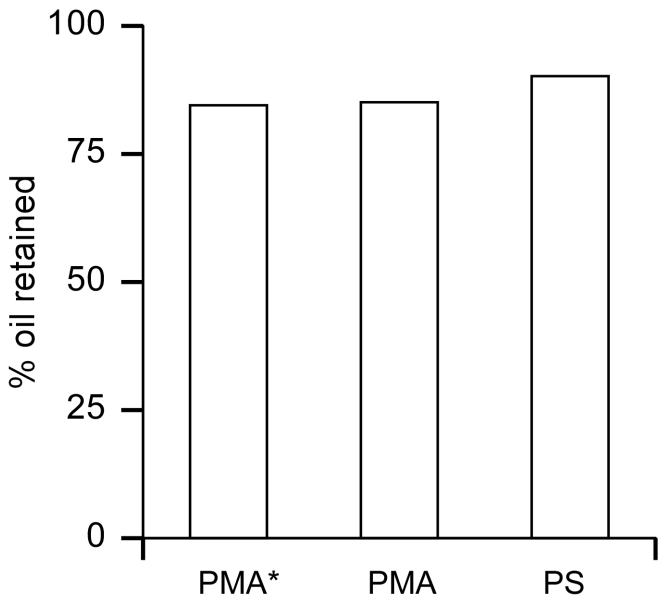


Figure 4.2: Screening by vegetable oil assay.

Comparison of oil retention quantified by Nile Red absorbance for PMA (JB20), imprinted PMA (JB21), and PS (JB8) resins.

PS (JB8/10) and PMA (JB20/21) resins were compared using algae lysed by pulsing or pressing, or unlysed algae. 1 mg of algae was loaded per 2 g resin, with binding results shown in Figure 4.3. In general, application of electric pulse or mechanical press did not result in a dramatic increase amount of oil retain bound on the resin. JB20 alone showed improved binding with pulsed (67.4% oil retention) and pressed (63.4%) lysis, over unlysed algae (52.2%). For the other three resins, oil retention either remained constant or decreased with lysis.

PS resin (JB8) bound more oil (88.9% oil retention with pressed algae) than PMA (JB20) resin (63.4%). Binding improved for PMA resin with corn oil imprinting, with 89.2% oil retention with pressed algae onto JB21, compared to 63.4% for JB20. Efforts to imprint PS resin actually led to a decrease in binding; 58.1% of oil was retained on JB10 with pressed algae, compared to 88.9% for JB8.

Preliminary studies focused on the relationship of polymer structure with oil accumulation, and monomers such as hexMA and HEMA were incorporated in PS and PMA resins to observe the effects of hydrophobic and hydrophilic moieties, respectively. However, it was observed that increasing the amount of algae passed through the resin column did not result in increase of total oil bound, as shown in Figure 4.4. Testing all resin formulations yielded the same general trend, and oil retention differences due to surface functional group could not be determined.

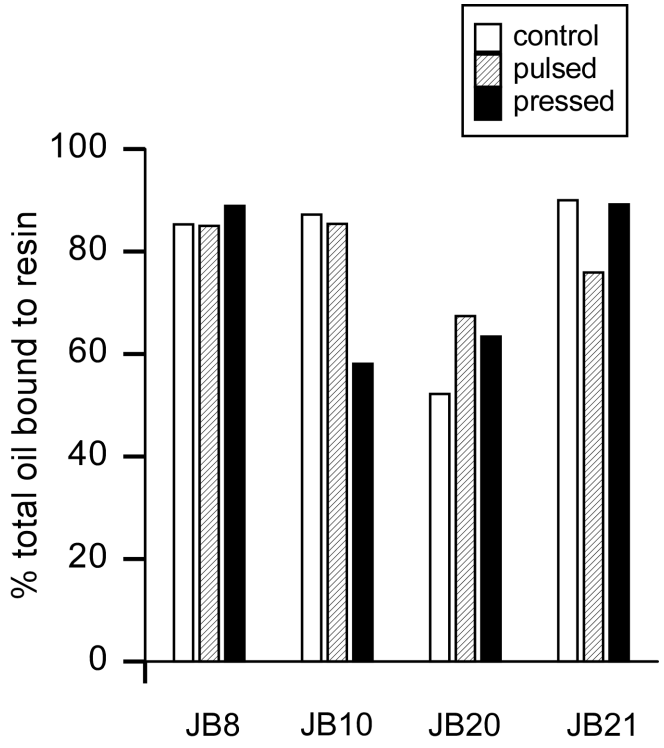


Figure 4.3: Algal oil binding assay for hydrophobic resins.

Comparison of algal oil retention for pulsed, pressed, and unlysed algae by PS (JB8), imprinted PS (JB10), PMA (JB20), and imprinted PMA (JB21) resins

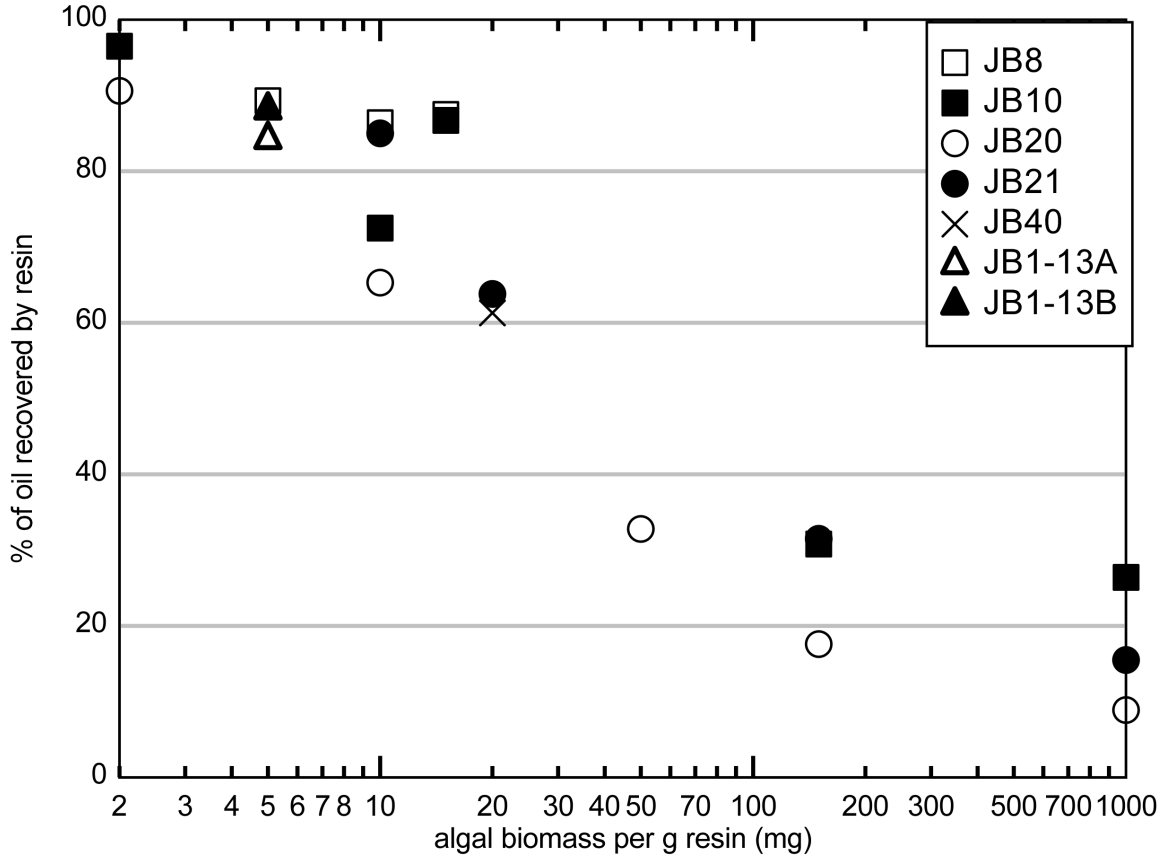


Figure 4.4: Algal oil binding assay for functionalized resins.

Oil accumulation for all resin structures as a function of algal biomass loading into the column. PS (JB8/10), PMA (JB20/21) resins, and DMAEMA (JB40) and hexMA (JB1-13A/B) functionalized resins.

Lipid class retention onto the resin was compared between DEG-functionalized PS resin (SN03) (Figure 4.5a) and PS resin (JB8) (Figure 4.5b). Oil eluted from SN03 consisted mainly of hydrocarbon, while JB8 primarily retained chlorophyll. Absolute yield of oil was equivalent between the two resin types.

In order to determine if oil from algae was saturating all the resin binding sites, algal suspension and corn oil were loaded either separately or as a mixture into the resin columns. Figure 4.6 shows that oil from algae bound 0.125 mg onto the resin (out of approximately 0.2 mg of algal oil available). For corn oil alone, 0.956 mg was bound out of 1 mg of total corn oil loaded. Applying a mixture of algae and corn oil resulted in 1.014 mg oil retention onto the resin.

Treatment of algal biomass with hot ethanol prior to loading through PS (JB8) and PMA (JB20) columns significantly improved oil accumulation onto the resin over no ethanol treatment (Figure 4.7a). JB8 bound 63.4% of algal oil after ethanol treatment, compared to 0.4% without treatment. Similarly, JB20 bound 73.8% of algal oil after ethanol treatment and 0.8% without treatment. Observation of cells by Sytox Green staining indicated that ethanol was effective at compromising the cell membrane even without application of heat, while heating alone without ethanol only slightly increased nuclear staining by Sytox Green (Figure 4.7b).

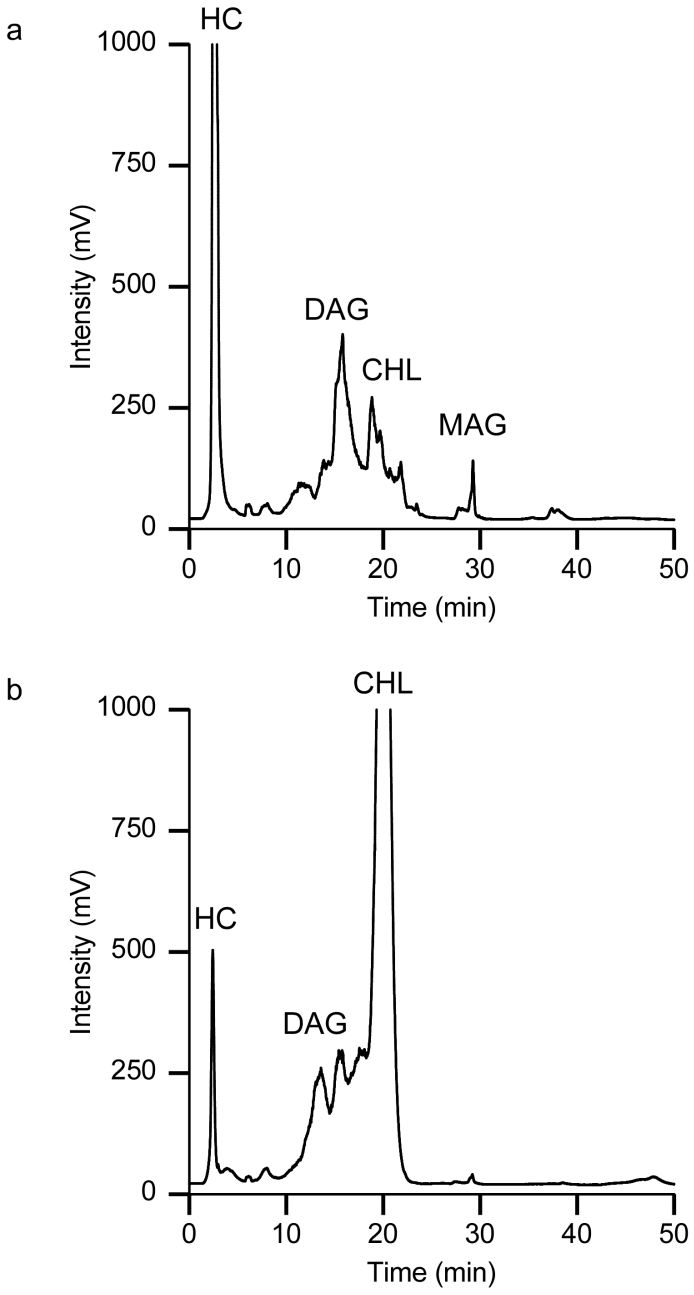


Figure 4.5: HPLC-ELSD chromatograms for oil retained on resin by DEG resin (SN03) (a) and PS resin (JB8) (b).

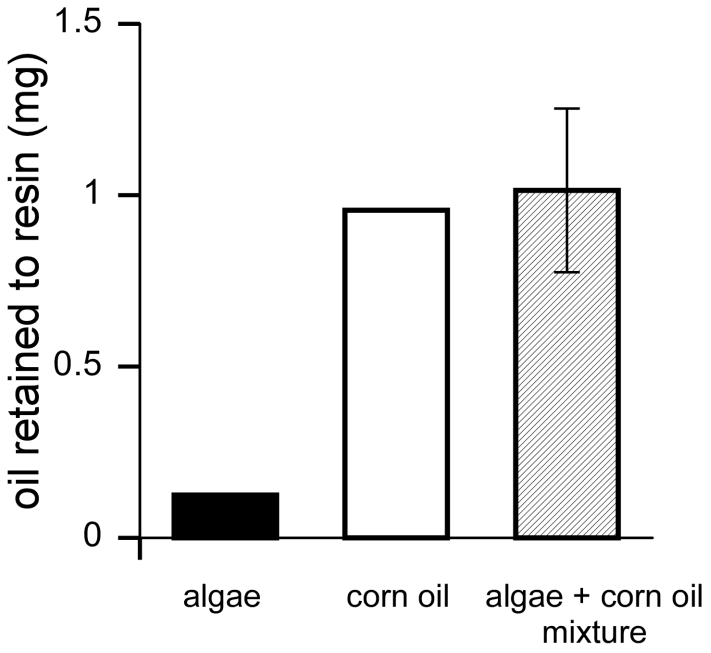


Figure 4.6: Comparison for algal and corn oil retention onto JB20.

Loading dye and corn oil mixture indicated higher binding capacity than demonstrated with algae alone.

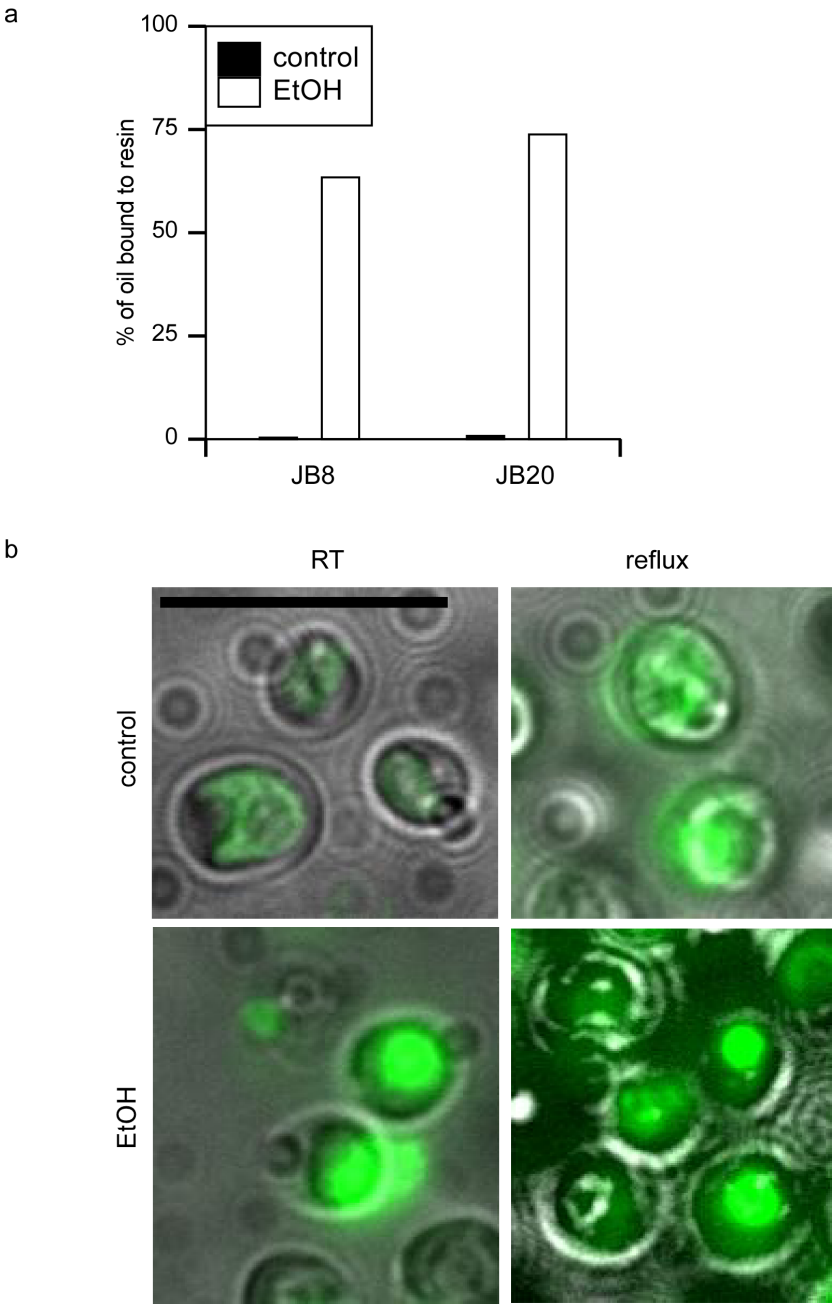


Figure 4.7: Treatment with ethanol on algal suspension.

Oil accumulation after algal biomass treatment with ethanol (a) comparison of ethanol treatment and control for PS (JB8) and PMA (JB20) resins (b) verification of cell membrane disruption by Sytox Green staining

4.4. DISCUSSION

Efficient extraction requires an absolutely dry biomass, but drying a large amount of harvested biomass requires time and energy. In order to improve biofuel economics, studies have focused on extraction methods that are effective on wet biomass (Belarbi et al. 2000; Johnson and Wen 2009; Griffiths et al. 2010; Beal et al. 2011; Wang and Wang 2011; Xu et al. 2011). Our approach differs in that it does not require dewatering the biomass to a paste, instead the algae can be extracted while in dilute suspension. Also, the extraction solvent is kept in a separate stream from the residual biomass, while other wet processing approaches attempt to extract by mixing solvent and paste.

We discovered that the problem of separating oil from wet biomass was technically challenging and not trivial. First, we speculated that simply lysing the cells so that the hydrophobic oils were exposed to the hydrophilic media would be sufficient to induce separation. Lysis was achieved on a lab-scale by freezing, sonicating, and heating. We also collaborated with CEM to lyse the cells by their electrodistention method. We verified cell lysis by using Sytox Green fluorescence microscopy and scanning electron microscopy. We vortexed the cell lysate suspensions with 10% toluene, and extracted the toluene for lipid analysis. However, we found that only nominal amounts of lipid could be recovered in this manner. Second, we attempted to precipitate the lipid from the algal lysates, either by generating soaps from the fatty acids and base, or using aluminum sulfate to bind to the lipids. In both cases, while successful with oil emulsions, the soaps and the aluminum sulfate would aggregate the entire algal cell instead of isolating the lipid. These tests suggested that after lysis, the majority of storage lipids still remain associated with the residual biomass.

After these unsuccessful trials, we proposed that hydrophobic polymers could act as solid phase sorbents to accumulate algal oil from suspension. We synthesized basic hydrophobic polymers composed of polystyrene and polymethacrylate by bulk polymerization for our tests. Adsorption of corn oil out of emulsion onto the hydrophobic polymers was very effective, as shown in Figure 4.2. Only about 10% of the oil introduced into the column remained in the aqueous suspension. In the end, total oil binding capacity could not be determined, since it appeared that with repeated loading the oil would continue to accumulate and would saturate the entire volume of the column.

We had theorized that the intracelullular storage lipids would only be accessible for resin interaction by first lysing the cell wall. The hydrophobic polymers were effective at oil binding when small amounts of algal biomass were loaded. However, this was achieved even without mechanical lysis (Figure 4.3). This led us to question whether surface oil was being bound from the algae, or perhaps the resins were able to lyse the algae. To test whether or not resin interaction was sufficient to lyse the algae, we agar plated unlysed algae that was passed through the column, but found that colonies were still viable. This suggests that only superficially available lipids were removed from the algae.

Increasing the amount of algae loaded did not result in a proportional increase in the amount of oil accumulated on the resin. Instead, the resins would saturate quickly, and the remainder algal oil was recovered in the residual biomass. As shown in Figure 4.4, increasing loading up to one gram of biomass per gram of resin resulted in less than 10% oil binding; considering the algae were typically comprised of 20% triacylglycerol by biomass, this would result in 20 milligram oil yield per gram of resin. This was surprising, since the polymers appeared to have much higher capacity in the oil emulsion studies. This again supported that only superficial lipids are accessible to the resins.

While a promising concept, molecular imprinting of the polymers did not appear to increase oil affinity of the lipids. This could be due to any of the following reasons: first, the triacylglycerol molecule could be too large to serve as a template when incorporated in the manner stated. Success with molecular imprinting of cholesterol has been described, though creation of the recognition site required polymerization with a covalently-bonded cholesterol template. The template is later removed to reveal the non-covalent binding site (Whitcombe et al. 1995). Second, oil-biomass interactions could inhibit any binding of the oil to the resin, regardless of binding site availability. This possibility was further explored with the ethanol treatment experiments.

Tests with mixed algae biomass and corn oil emulsions showed that the corn oil could be easily recovered out of the biomass, as shown in Figure 4.6. Even though algal oil binding was limited to 1 mg per g of resin, corn oil was still bound to a capacity greater than 1 g per g resin. This indicated that even when lysed, the resin cannot separate the algal oil from biomass. This problem is not completely unexpected; preliminary work had focused on separating oil from lysed algae based on immiscibility of oil with water, or by precipitation of fatty acids with alkali soaps. Unfortunately, these attempts were not successful at recovering high oil yields, and it was determined by Nile Red staining that lipids remained with the algal lysate.

Instead, we observed that the functional group may confer some lipid species selectivity. Testing the DEG resin (SN03) against a standard PS resin (JB8), it was found that SN03 was more selective for hydrocarbon species than JB8 (Figure 4.5). In fact, this was apparent from visual inspection of the oil elution, since the SN03 eluate was yellow from hydrocarbon (specifically, *beta*-carotene) and JB8 eluate was green from chlorophyll. Unfortunately, variation of functional group did not result in change in overall binding capacity. This suggests that other materials may be bound to the resin.

Based on similar experiments conducted with bacteria, the algae were treated with ethanol in order to disrupt the cell membrane (Ingram 1981). As shown in Figure 4.7, ethanol treatment, both with and without heating, was able to disrupt the cell membrane so that it was permeable to the indicator Sytox Green. Heating alone at the given conditions was not sufficient to lyse the cells.

Not only were the cells lysed, the amount of oil bound to the hydrophobic resins also increased dramatically, indicating that ethanol transferred the oil between the aqueous media and the hydrophobic resins. In a similar study, combination of sonication and hot ethanol treatment was used in order to disrupt algae and separate lipids without prior biomass drying (Wang and Wang 2011). After centrifuging the treated biomass, they were able to separate lipid and residual biomass, and recover nearly 100% of available lipid. As an improvement, our method would eliminate energy-intensive centrifugation by collecting the extracted lipid onto the resin. The combination of low energy lysis, alcohol-mediated transfer, and hydrophobic resin technology may be an effective extraction strategy.

4.5. CONCLUSION

A solventless oil extraction method can improve biofuel process economics by eliminating solvent contamination of the residual biomass. Hydrophobic polymers can bind oil out of aqueous emulsion, but have limited capacity when binding intracellular lipids from algae. Several approaches were used to improve the binding affinity of the polymers for algal oil: resins were molecularly imprinted with triacylglycerol binding sites during polymerization, resins were functionalized with either hydrophobic or hydrophilic surface groups, and algal biomass was pretreated with dilute ethanol prior to

resin loading. Ethanol pretreatment alone was successful, since it disrupted interactions between the oil droplets and the biomass and allowed hydrophobic polymers to easily recover the lipids. Further work must be done to develop resins with the same cell disrupting capability and greater affinity for the lipids.

Chapter 5: Application of Amberlite anion exchange resin for one-step biodiesel synthesis from algae

5.0. ABSTRACT

Microalgae are particularly attractive as a renewable feedstock for biodiesel production since they grow rapidly without necessarily encroaching on cropland. At present, processing algae can be more expensive than land crops because of the need to process large volumes of dilute algal suspensions. In an effort to circumvent this problem, we have explored the use of resins as a means for simplifying the processing of algae to biofuel. Anionic exchange resin can bind and accumulate algae cells out of suspension to form a dewatered concentrate, which can be immediately treated with transesterification reagents to yield biodiesel. Here we study the efficiency of a one-step, *in situ* transesterification and the reusability of the resin substrate.

5.1. INTRODUCTION

Biodiesel formed from glycerolipids is a renewable energy source that is compatible with current diesel engines and commercial fuel supply infrastructure (Scott et al. 2010). It has a relatively high energy density, making it one of the most attractive options among alternative energy sources (Durrett et al. 2008). It also more environmentally friendly in terms of producing lower exhaust emissions (Durrett et al. 2008), and having a near-neutral carbon footprint (Gerpen 2005). Biodiesel production is an established process, having been in use for the past 50 years, but its large-scale production suffers for a lack of plentiful oil feedstock (Chisti 2007). Of the high oil-producing plant feedstocks, algae are an attractive source since some algae make

relatively large amounts of triacylglycerols (Li et al. 2008) and use of algae for fuel would not impact the food supply (Bastianoni et al. 2008).

Biodiesel is produced by acid- or base-catalyzed transesterification of glycerolipids to give fatty acid methyl esters (FAMEs) and glycerol (Schurchardt et al. 1998). However, water interferes with the conversion, resulting in hydrolysis or saponification (Schurchardt et al. 1998). Soap forms emulsions that interfere with downstream biodiesel processing (Serio et al. 2008). Thus water must be removed prior to transesterification.

Algae grow at dilute concentrations, generally less than 1 g/L (Li et al. 2008). To extract the oil, algae must first be harvested and then dried before the useful oil can be extracted. Drying large amounts of algae biomass by oven is costly and can represent 69% of total processing costs (Sander and Murthy 2010). After drying, lipids are extracted with solvents before the resulting oil is converted to biodiesel. As a consequence, development of large-scale biodiesel production from algae has been prohibitively expensive (Sander and Murthy 2010).

In an effort to develop cost effective methods for processing algae we have explored the uses of resins to streamline the harvesting and drying steps. Microalgae have negatively charged cell surfaces that bind tightly to anion exchange resins such as Amberlite CG-400, a styrene-based resin with fixed positive charges. Previously, algal powders have been immobilized onto Amberlite in order to improve metal uptake during wastewater treatment (Onyancha et al. 2008). However, the use of Amberlite to bind and concentrate live algae from dilute suspensions has not been reported. Here we show that Amberlite provides a simple way to concentrate and dewater algae. Furthermore, treatment of the resin with methanol / sulfuric acid regenerates the resin while at the same time efficiently converting the saponifiable fatty acids to FAME.

5.2. MATERIALS AND METHODS

Ethanol, hexane, methanol ethyl acetate isopropanol, issoctane and 2-ethoxyethanol (2-EE) (Acros, ACS grade) were obtained from Fisher and were ACS grade or better. Sulfuric acid and potassium hydroxide were reagent grade. Sodium methoxide in methanol (0.5M) was obtained from Sigma-Aldrich (St Louis). Methyl oleate and methyl palmitate (Sigma-Aldrich), triolein (Nuchek Prep, Elysian, MN) and mineral oil (Squibb) were used as lipid standards.

All HPLC solvents were degassed and filtered through 0.5 micron PTFE filters (Ominpore, Waters Corp) prior to use. Isooctane was dried by storing over calcium hydride and filtering before use.

Amberlite® ion exchange resin CG-400 (100-200 mesh, chromatographic grade; Mallinckrodt, St. Louis, MO) was prepared by washing and settling in distilled water in order to remove fines, then dried at 55°C.

5.2.1. Algal cultivation and harvest

For these studies, *Neochloris oleoabundans* (UTEX LB 1185) was obtained from The University of Texas at Austin Algal culture collection and KAS 603, a saltwater species of *Chlorella*, was provided by Kuehnle Agro Systems (Hawaii).

Neochloris was cultivated in freshwater Bold 3N medium using an airlift bioreactor illuminated with cool white fluorescent lights on a 12:12 light:dark cycle and aerated with ambient air using an oil-free diaphragm pump (Brown and Bold 1964). KAS 603 was cultivated in the same way using f/2 saltwater medium (Jeffrey and LeRoi 1997). For nitrogen starvation experiments, *Neochloris* was grown for 14 days in Bold 3N media, harvested by centrifugation, resuspended in sodium nitrate-free Bold 3N, and

cultured for an additional 21 days. For resin binding and FAME synthesis, algae were harvested by centrifugation and resuspended in distilled water to concentration of 0.4 g/L.

5.2.2. Algal mass quantification

Routine determination of algal dry cell weight (DCW), obtained by measuring the optical density of chlorophyll at 680 nm (OD_{680}) using a Shimadzu spectrophotometer. The conversion of OD_{680} to DCW was accomplished by generating a dilution series for each species, recording the OD_{680}, and then collecting dilutions onto pre-weighed cellulose acetate membranes (Pall Co., Port Washington, NY), which were then dried in a vacuum oven (15 in. Hg., 60 °C) for 12 h before obtaining the final weights. To avoid optical filtering effects, samples were diluted, if need be, such that the OD_{680} was always less than 1.8.

5.2.3. Algal binding and FAME generation

For algae binding and FAME conversion studies, 10 ml polypropylene columns that has been washed with 1M hydrochloric acid and then distilled water were loaded with 2 g of Amberlite resin. To load the resin with algae about 50 mg (a slight excess) based on the OD_{680} of the algae suspension was passed through the resin. The OD_{680} of the flow through was then measured to determine the DCW of algae bound to the resin.

For transesterification and elution of algae from the resin, excess water was removed from column by vacuum and followed by the addition of 100 mL of transesterification reagent. Reagents tested consisted of ethanol or methanol containing either 5% w/v sulfuric acid or 5% w/v potassium hydroxide, or 0.1 M sodium methoxide in methanol. The eluate was collected and the transesterification reaction continued at room temperature for 12 h. FAME was extracted by partitioning the eluate into organic and aqueous phases with 20 ml of hexane and 20 mL of distilled water using a separatory

funnel. The extraction was repeated with a second 20 mL portion of hexane. The combined extracts were then dried by rotary evaporation and resuspended in 1 mL hexane:isopropanol (3:1, v/v) for HPLC analysis.

For comparison, FAME was generated directly from dried algal biomass, based on a method described previously (O'Fallon et al. 2007). Algal pellets containing approximately 30 mg algae in preweighed tubes were dried using a SpeedVac concentrator (Savant) for 12 h at 10^{-1} Torr with heating. Final tube weight was then measured to obtain an accurate algal DCW. The dried algal pellet was suspended in methanol, transferred to a glass centrifuge tube where the methanol was brought to a final volume of 5.3 mL. Lipids were then saponified by adding 0.7 mL of 10 N potassium hydroxide and heating at 55 °C for 1.5 h. Once the samples cooled, lipids were re-esterified by adding 0.6 mL of concentrated sulfuric acid and heating again at 55 °C for 1.5 h. After cooling, FAME was extracted with 2 mL of hexane using a centrifuge to force the separation of the mixture into two layers. The upper hexane layer was then transferred to vial for HPLC analysis.

Phosphate detection was conducted by thin layer chromatography on aluminum-backed silica plates. Plate was developed by (1) chloroform:methanol:acetone:acetic acid:water (65:10:20:10:3, v/v/v/v/v) (Kinsey et al. 1980) then (2) hexane:diethyl ether:acetic acid (90:10:1, v/v/v) (Suzuki et al. 1969). Unsaturated lipids were visualized by iodine vapor, and phosphates were visualized by molybdate stain (Skipski et al. 1962).

5.2.4. Total lipid extraction from algae

Total lipid was determined by method in Jones et al (in review) (Jones et al. 2011). Dried algal pellets (approximately 30 mg) were extracted with 20 mL of 2-EE for 30 min at 60 °C with continuous stirring. The solution was then filtered through 0.47 μm

PTFE membrane (Millipore) and the residual biomass was extracted with a second 20 mL portion of 2-EE. After filtration, the two filtrates were combined, dried under vacuum, and weighed.

5.2.5. Quantification and characterization by HPLC-ELSD/MS

Lipid composition and FAME content were analyzed using an HPLC (Surveyor LC Pump and Autosampler Plus, Thermo Finnegan) coupled to both evaporative light scattering detector (ELSD; Sedere Sedex 75) and quadrupole mass spectrometer (Thermo Finnegan MSQ) using a 10:1 line splitter (Analytical Instruments). Xcalibur software controlled operation of the autosampler, pump, and mass spectrometer. ELSD analog data was acquired through an A/D data acquisition box (Agilent Technologies, SS420X) and RS232 PCI data acquisition card (Sea Level Systems, 7406S).

Lipid standards and extracts were resolved using a normal-phase poly-vinyl alcohol-bonded silica column (YMC Pack PVA-Sil-NP, 250 mm x 4.6 mm I.D., 5 μm bead size) protected by a Waters Guard Pak™ guard column containing Nova-Pak™ silica inserts. Mobile phase consisting of isooctane, ethyl acetate, and isopropanol:methanol:water (3:3:1) + 0.1% acetic acid was used with a gradient sequence specified in Table 5.1. ELSD was run at 30 °C at gain setting 8. Mass spectrometer was run in APCI positive mode with probe temperature of 400 °C.

Identity of extract components was determined by comparison with retention time of lipid standards. Lipid class identity was further confirmed by spiking the sample with a known amount of standard to observe proportional increase in ELSD signal. Further information about fatty acyl constituents of FAME was obtained by the APCI-MS mass fragmentation patterns.

time (min)	flow rate (mL/min)	A (%)	B (%)	C (%)
0	1.5	100	0	0
5	1.5	98	2	0
15	1.5	75	25	0
19	1.5	20	80	0
24	1.5	0	100	0
32	1.3	0	50	50
38	1.0	0	15	85
43	1.0	0	0	100
52	1.0	0	100	0
54	1.0	0	100	0
60	1.5	90	10	0
64	1.5	100	0	0
74	1.5	100	0	0

Table 5.1: HPLC mobile phase gradient method.

Three-solvent system of iso-octane (A), ethyl acetate (B), and isopropanol:methanol:water (3:3:1, v/v/v) + 0.1% acetic acid (C) (Jones et al. 2011).

5.3. RESULTS

Figure 5.1 illustrates the two stages of *in situ* biodiesel synthesis. The algae were bound to the Amberlite resin; clear growth media is passed through the column, and can be directly reused for algal cultivation (5.1a). The resin was regenerated by eluting the column with transesterification reagent, removing algae from the resin while simultaneously derivatizing the algal lipids into esters (5.1b).

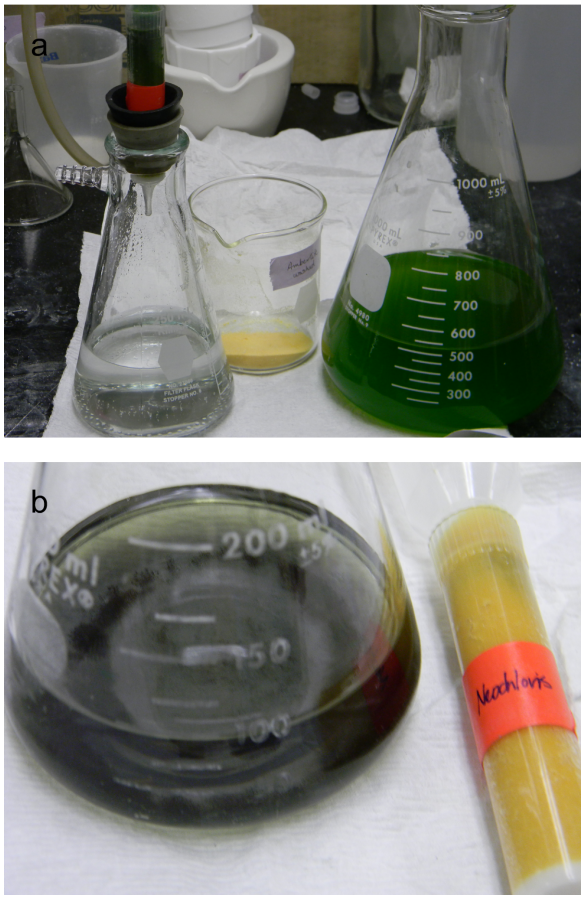


Figure 5.1: Resin-bound transesterification.

Binding algae onto an Amberlite resin column results in clear media eluate (a); elution of resin column with transesterification reagent regenerates the column and directly derivatizes algal lipids (b).

For quantification of algal mass bound to the resin, chlorophyll optical density calibrations that related concentration of algae (DCW/ml) to absorbance at 680 nm were determined for both algal species. As shown in Figure 5.2, the calibration fits were linear over the optical density range tested ($R_{Neo\text{-}heal}^2 = 0.997$, $R_{Neo\text{-}str}^2 = 0.994$, $R_{Chl}^2 = 0.999$).

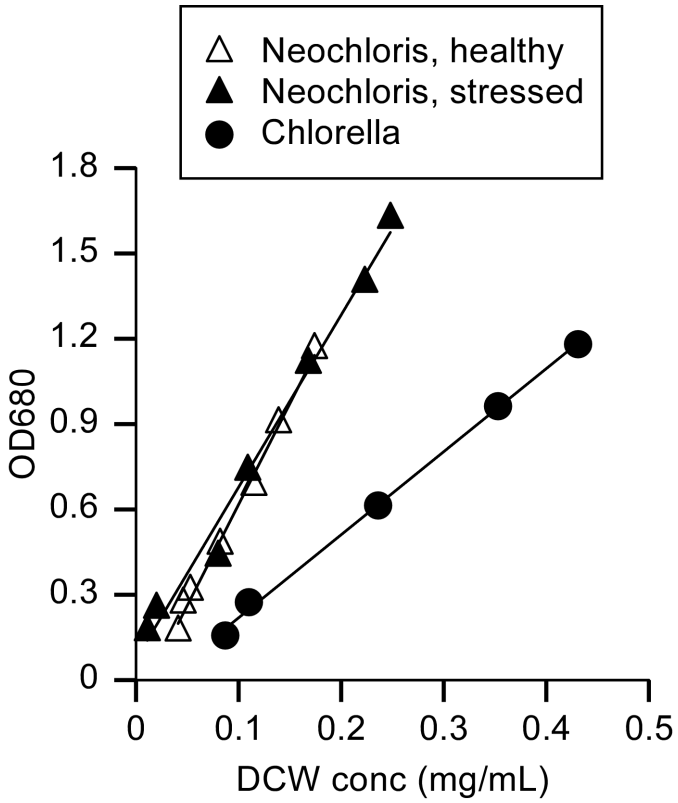


Figure 5.2: Algal biomass quantification.

Chlorophyll optical density calibration standard at 680 nm as a function of healthy *Neochloris*, stressed *Neochloris*, and *Chlorella* DCW concentration

HPLC retention times for HC, FAME, and TAG were determined from lipid standards as shown in Figure 5.3a. Normal-phase HPLC has been demonstrated to separate lipid classes (Nordback et al. 1998), and retention time differences due to fatty acyl chain type are small enough that lipid classes appear as a single quantifiable peak, such as observed in thin layer chromatography. With normal-phase HPLC, mineral oil, the standard for HC, was quickly eluted (RT_{HC} = 2.48 min), one minute after the column void volume (T_0 = 1.46 min). As the fraction of ethyl acetate in the mobile phase increases, FAME is eluted (RT_{FAME} = 5.29 min) followed by TAG (RT_{TAG} = 10.20 min). Quantification of the lipid classes was based on calibration fits relating ELSD signal peak area to known standard concentrations (Figure 5.3b). Calibration was fit to second-order polynomial (R_{HC}^2 = 0.994, R_{TAG}^2 = 0.999, R_{FAME}^2 = 0.997), as ELSD response is nonlinear (Dreux et al. 1996). HPLC-ELSD was used to quantify FAME yield, as shown in Figure 5.4a, and original algal TAG content, as shown in Figure 5.4b-d.

Further information on fatty acyl substituents in transesterification products were obtained from positive mode APCI-MS. Mass spectra of FAME standards yielded regular patterns including protonated molecular ion $[M+H]^+$ and fragment formed by loss of methoxy group $[RCO]^+$ that was seen in unsaturated species (Figure 5.5). These patterns were used in order to identify FAME species in the algal transesterification product. Fatty acyl composition was determined by abundance of the protonated molecular ion peaks at m/z 243 (C14:0), 271 (C16:0), 293 (C18:3), 295 (18:2), 297 (C18:1), 299 (C18:0), 327 (C20:0), 333 (C21:4), and 359 (C23:5).

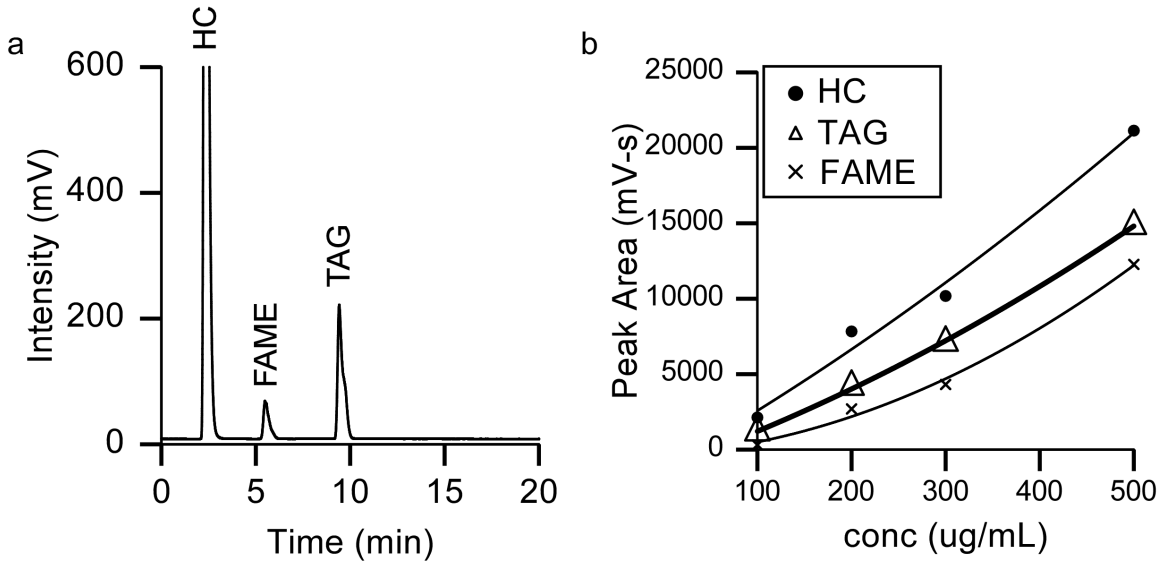


Figure 5.3: HPLC chromatograms for lipid quantification.

Retention standards for hydrocarbon (HC), fatty acid methyl ester (FAME), and triacylglycerol (TAG) (a), ELSD calibration curve for quantification of nonpolar lipid classes HC, FAME, and TAG (b).

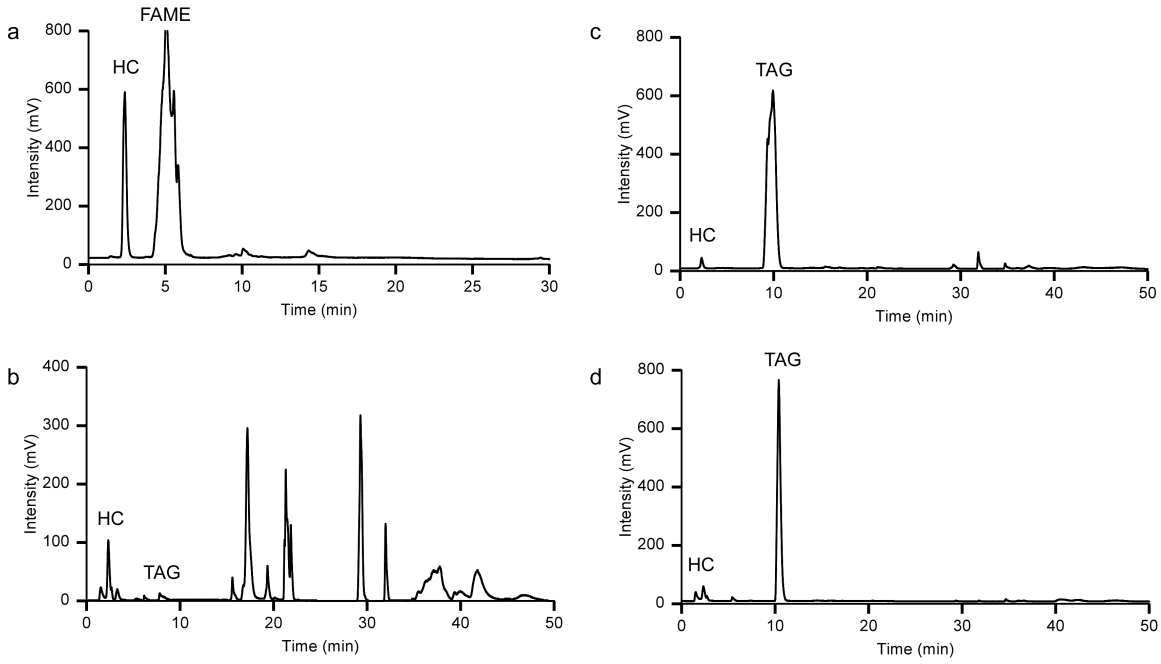


Figure 5.4: HPLC chromatograms for lipid quantification.

Typical hexane extract from algae column elution shows algal HC and FAME recovered after transesterification (a), neutral lipid profiles for healthy *Neochloris* (b), stressed *Neochloris* (c), and *Chlorella* (d).

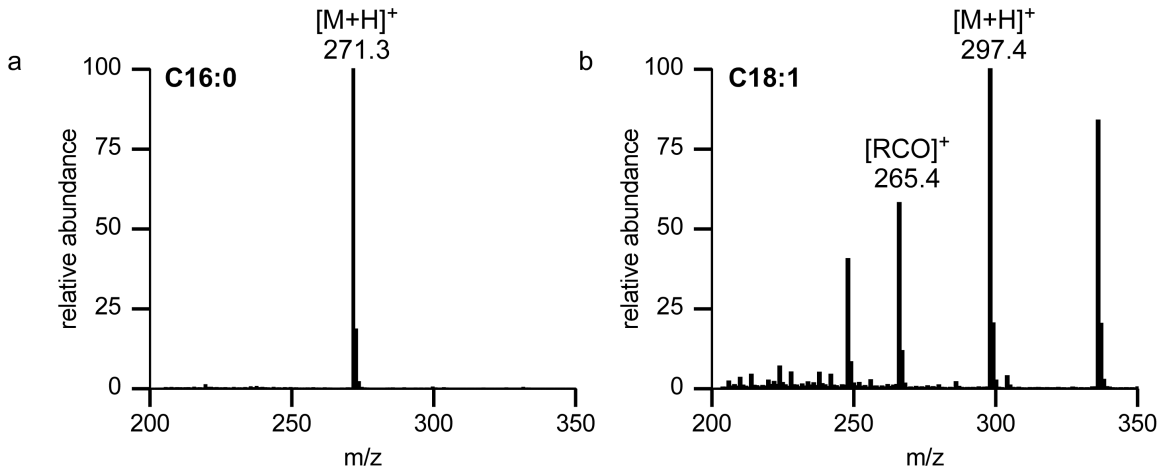


Figure 5.5: Mass spectra of FAME standards.

Positive mode APCI-MS spectra for standards methyl palmitate (a) and methyl oleate (b) show protonated molecular ion $[M+H]^+$ and fragment ion $[RCO]^+$.

The reusability of alga-binding resin and transesterification reagent was determined by four cycles of binding and elution for *Neochloris* (healthy) and *Chlorella* using fresh 5% sulfuric acid / methanol as the eluent for each cycle. For *Neochloris*, the results show that there was no loss of binding capacity over four cycles of resin loading and elution. Amberlite bound 37.0 mg of *Neochloris* per g of resin and 12.8 mg *Chlorella* per g resin (Figure 5.6a), and was able to bind subsequent *Neochloris* batches at nearly 100% of its initial binding capacity (Figure 5.6b). For *Chlorella*, there was an initial 40% loss in binding capacity after the first cycle but binding capacity remained constant for the subsequent three cycles.

Since both the methanol and sulfuric acid in the eluent were seemingly present in large excess, a set of cyclical binding and elution experiments were carried out similar to those above only here, the initial 5% sulfuric acid / methanol solution was reused for subsequent binding and elution cycles. Figure 5.6b shows that reusing the transesterification reagent affect algae binding capacity of the resin or the amount of algae eluted. The same trend was also observed when eluting with methanol containing sodium methoxide. However, reusing the reagent tended to decrease the FAME yield, since sulfuric acid was consumed in each cycle.

Conversion of algal lipids into FAME was compared between resin-bound algae and dry algal pellets for both healthy and nitrogen-deprived *Neochloris*, and *Chlorella*, as shown in Figure 5.7. Base hydrolysis followed by acid re-esterification of dry pellets resulted in 21.7%, 20.9%, and 35.2% of dry weight recovered as FAME for healthy *Neochloris*, stressed *Neochloris*, and *Chlorella* respectively. Acid-catalyzed transesterification of resin-bound algae resulted in 13.6%, 6.9%, and 37.6% of dry weight recovered as FAME for healthy *Neochloris*, stressed *Neochloris*, and *Chlorella*

respectively. For comparison, FAME synthesis yields are shown along side total lipid extract amounts. Crude lipid extract constituted 31.4% of total dry weight for healthy *Neochloris*, 35.4% for stressed *Neochloris*, and 41.3% for *Chlorella*. HPLC analysis of the total lipid extract determined that TAG constituted 0.4%, 8.6%, and 11.7% of dry weight for healthy *Neochloris*, stressed *Neochloris*, and *Chlorella*, respectively.

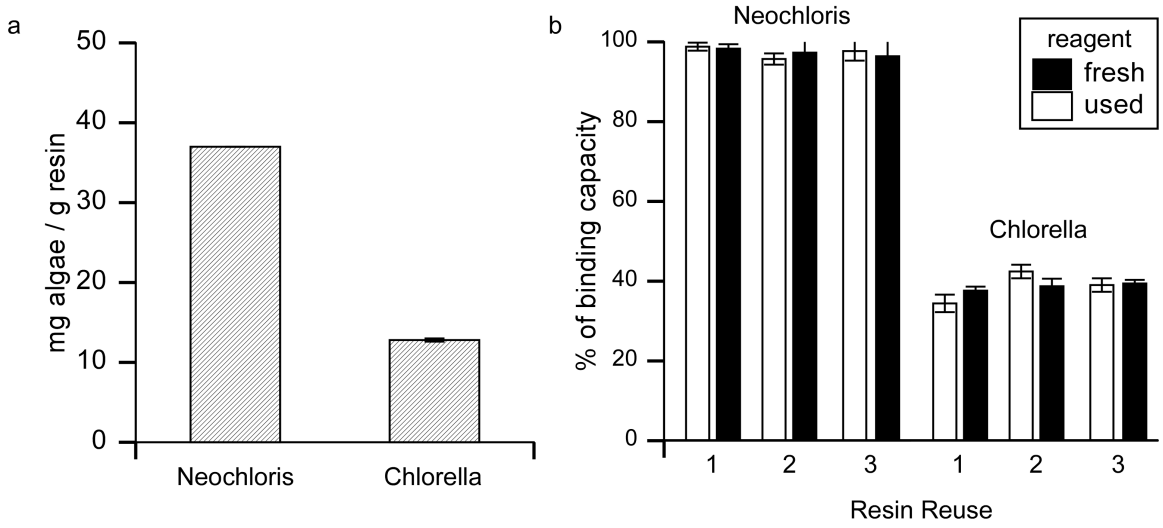


Figure 5.6: Binding capacity of Amberlite.

Comparison between *Neochloris* and *Chlorella* (a), comparison of *Neochloris* and *Chlorella* binding capacity of the resin for four loadings (initial binding and three reuses) eluted with fresh or reused transesterification reagent (b). Bars represent average of three replicates, brackets indicate standard error of the mean.

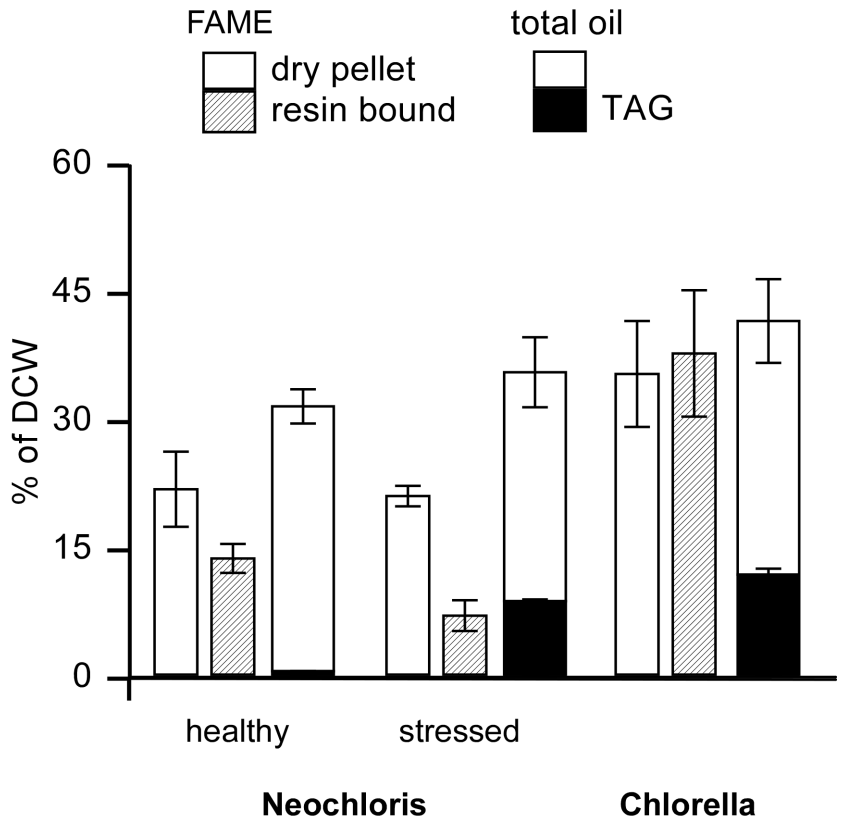


Figure 5.7: Transesterification of resin-bound algae and dry algal pellet.

Comparison of FAME generation by acid transesterification of resin-bound algae and base-acid transesterification of dry algal pellet for *Neochloris* (healthy and stressed) and *Chlorella* algal species; yields from transesterification products are compared to initial TAG and crude oil content available in the algae. Bars represent average of three replicates, brackets indicate standard error of the mean.

Fatty acyl groups in FAME were identified and quantified by APCI-MS, as shown in Table 5.2. The composition of C14:0, C18:1, and C23:5 FAME was nearly equivalent among the three algal groups tested. In addition, *Neochloris* had slightly more C18:2 than *Chlorella*. Figure 5.8 illustrates a trend observed in some saturated and unsaturated FAME species. Healthy *Neochloris*, having lower TAG content, yielded more C18:3 and C21:4 species and less C16:0, C18:0, and C20:0 than the other two algal groups. In contrast, stressed *Neochloris* and *Chlorella*, having higher TAG, yielded more C16:0, C18:0, and C20:0, and less C18:3 and C21:4 than healthy *Neochloris*.

The efficacy of resin-bound transesterification was compared between methanol and ethanol. As shown in Figure 5.9, acid-catalyzed transesterification of resin-bound *Neochoris* with methanol recovered 10.3% as FAME whereas with ethanol, 12.4% was recovered as fatty acid ethyl esters (FAEEs). Resin-bound transesterification of *Chlorella* recovered 49.0% of DCW as FAME and 39.4% of DCW as FAEE.

Base-catalyzed transesterification was also tested on resin-bound algae, but FAME generation was low. Potassium hydroxide / methanol elution did not result in FAME generation, while sodium methoxide / methanol resulted in incomplete reaction. *Neochloris* yielded 1.4% of DCW as FAME, and *Chlorella* yielded 6.6%.

Hexane and chloroform extractions of eluate did not show the presence of phosphates by molybdate test (Figure 5.10a), while the assay indicated that phosphates were present in the aqueous phase of the eluate (Figure 5.10b).

FA	m/z	healthy *Neochloris* (%)	stressed *Neochloris* (%)	*Chlorella* (%)
C14:0	243	2.9	2.7	2.6
C16:0	271	16.3	41.4	50.6
C18:3	293	22.2	9.7	8.3
C18:2	295	2.7	2.4	0.6
C18:1	297	2.3	3.9	2.7
C18:0	299	4	9.2	13.3
C20:0	327	0.9	1.8	9.0
C21:4	333	46.0	26.2	10.4
C23:5	359	2.6	2.8	2.4

Table 5.2: FAME molecular ion assignments and relative abundance for healthy and stressed *Neochloris*, and *Chlorella*.

Values are based on averages of three replicates.

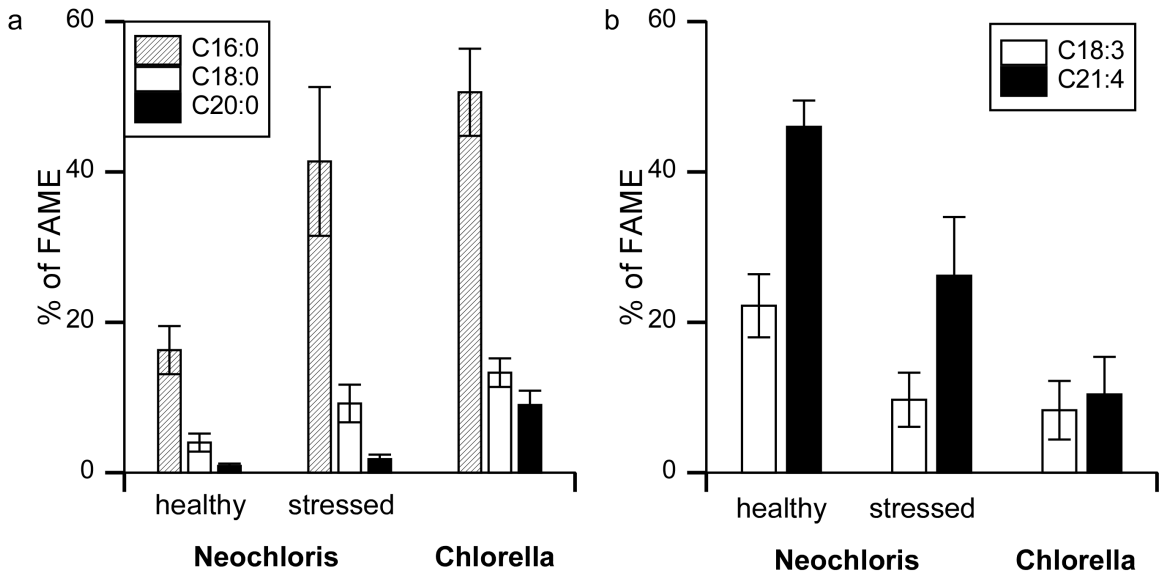


Figure 5.8: Comparison of fatty acyl substituent in FAME.

Abundance of C16:0, C18:0, and C20:0 (a), and C18:3 and C21:4 FAME (b) in healthy and stressed *Neochloris*, and *Chlorella*. Bars represent average of three replicates, brackets indicate standard error of the mean.

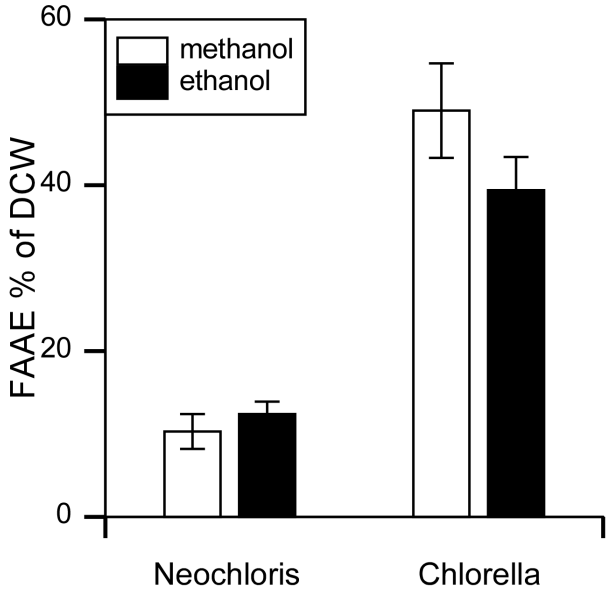
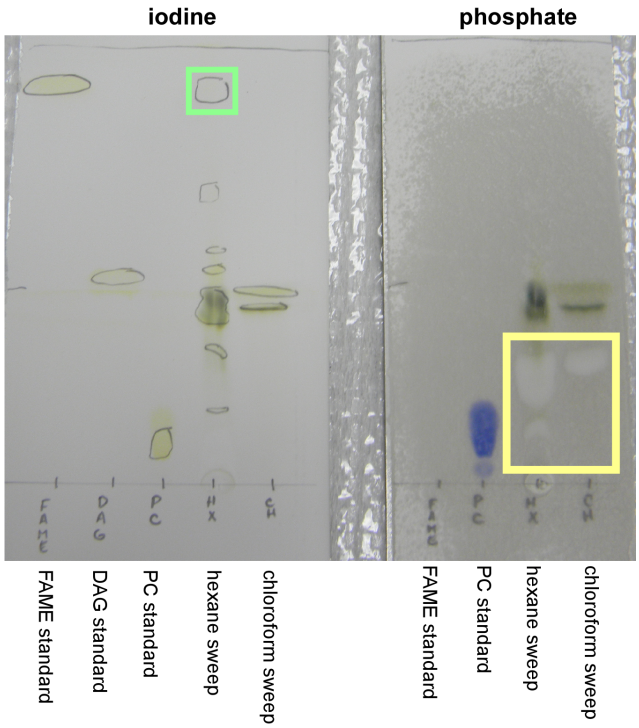


Figure 5.9: Alternate transesterification reagent.

Comparison of transesterification yield relative to DCW for methanol and ethanol with sulfuric acid catalyst for resin-bound *Neochloris* and *Chlorella*. Bars represent average of three replicates, brackets indicate standard error of the mean.

a



b

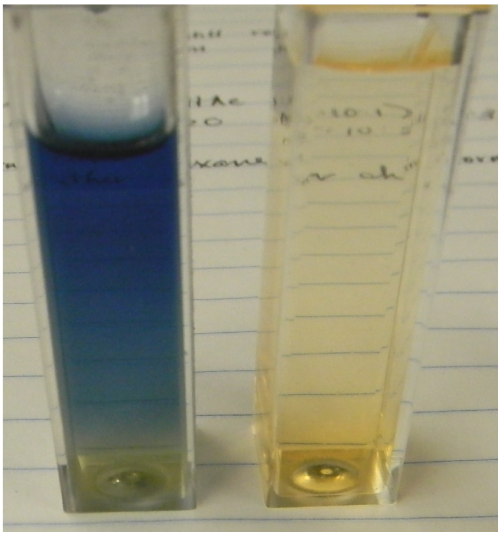


Figure 5.10: Phosphomolybdate test of eluate.

TLC plate of hexane and chloroform extracts indicate the prescence of FAME and pigment groups by iodine stain, but no phosphate groups, as marked by the yellow box (a); aqueous phase of eluate was positive for phosphates (b).

5.4. DISCUSSION

Amberlite containing strongly basic, quaternary ammonium groups on a polystyrene resin was shown to bind and concentrate two different species of algae (Onyancha et al. 2008). As expected, binding capacity was reduced in high ionic strength media, since anions compete for binding sites. This would suggest that Amberlite is best used with freshwater algae (salinity 5 psu). Even when controlling for media environment, different binding capacities were observed for *Neochloris* and *Chlorella*. This indicated that differences in cell surface chemistry freshwater and marine *species* can also affect binding behavior. We also found that resin reusability varied between *Chlorella* and *Neochloris* but the reason for this difference is not known.

Reusing the transesterification reagent was as effective as using fresh reagent for removing algae from the resin and synthesizing FAME. While the base reagent were able to elute algae off the resin, yields of FAME were greatly reduced, perhaps due to greater sensitivity to residual water.

Traditionally, gas chromatography has been used for FAME analysis (Tornabene et al. 1983). Here we found that normal-phase HPLC/MS was useful for rapid characterization of the entire FAME fatty acid profile as well as any unreacted TAG, such that one can determine the completeness of the reaction. HPLC also detected HC in the hexane extract, which likely explains the difference between the total lipid assessed gravimetrically and the amount that can be recovered as FAME. While often considered a lesser component of green algal lipids, it was often observed that HC contributed a significant signal peak in FAME extracts (Figure 5.4a).

Comparable FAME yields for *Chlorella* were obtained between resin-bound one-step transesterification at room temperature and dried pellet acid-base transesterification

at 55 °C, while resin-bound conversion of *Neochloris* yielded approximately 60% of the FAME generated from the dried pellet. While heated synthesis was completed in 3 hours compared to 12 hours for room temperature, consider that, at the commercial-scale, it may be less cost efficient to heat a large volume of biomass rather than to let it sit for a longer period of time.

These studies show that considerably more FAME is generated than can be accounted for strictly from TAG. This is especially true for *Chlorella* where 10% of the DCW was present as TAG but nearly 40% of the DCW recovered as FAME. This indicated that lipid components other than neutral lipids must be contributing to FAME derivatization, *e.g.*, pigments, glycolipids, and phospholipids. While one molecule of TAG is converted to three molecules of FAME, one molecule of glycolipid or phospholipid is converted to only two molecules of FAME. Thus, complete reaction does not mean there is a weight-for-weight conversion of biomass, as some of the original crude lipid extract cannot be recovered in the biodiesel product.

One of the surprising results of this study is the finding that *Neochloris* accumulates high amounts of TAG when subjected to nitrogen deprivation (Tornabene et al. 1983), but with only a modest increase in total lipid. While TAG increased nearly ten-fold, total lipid still constituted approximately 20% of dry weight, and FAME yield from dried biomass was comparable between healthy and stressed *Neochloris*. The most notable difference between FAME generated from healthy and stressed *Neochloris* was found in the fatty acid composition. As TAG becomes a greater proportion of total lipid, the abundance of saturated species increased, while unsaturated species decreased. Similarly, *Chlorella*, which has a greater proportion of TAG, also has relatively high level of saturated species.

In order to determine the versatility of resin-bound transesterification, we tested different elution reagents. We found that ethanol and methanol were equally effective at eluting algae off the resin and ethanol was nearly as effective in the transesterification. .Because of its low cost and wide availability, methanol is often used for biodiesel synthesis (Gerpen 2005). However, ethanol cost may decrease in the future as supply increases with advancements in bioethanol.

Even though Amberlite had a lower binding capacity for *Chlorella*, since it has high TAG and total oil content, it yielded as much FAME per g resin as *Neochloris*, approximately 5 mg of FAME per g of resin. However, the quality of crude biodiesel produced by direct transesterification of algae must be considered. MS spectra suggest that healthy *Neochloris* yields larger molecular weight, unsaturated FAME species. These would have high melting point and iodine values, and would be unsuitable for direct biodiesel use. However, they have great potential as high-value, nutritional feedstock. While nutrient-deprivation requires an extra step during cultivation of *Neochloris*, it yields more TAG and thus more saturated FAME. Likewise, *Chlorella* generally has high level of TAG, and so is an ideal feedstock for biodiesel.

We used Amberlite for these initial studies because it was readily available. However, Amberlite exhibits a rather low binding capacity for algae, which gets substantially worse when one goes from fresh water to saltwater. During the course of these studies, we have synthesized resins that exhibit 20-fold better binding capacities in both fresh and saltwater. Thus we anticipate with the development of better resins, much better overall performance will be obtained.

5.5. CONCLUSION

While transesterification of algal oil is an established method for producing FAME, the high cost of dewatering biomass is an obstacle to the widespread adoption of biodiesel. We have presented a low cost method for concentrating biomass that allows direct transesterification of biomass, negating the need for a separate oil extraction step. Our studies with Amberlite suggest that resin-based methods can be a cost effective means to decrease algal biodiesel production cost. However, there are several ways in which the application of resins can be improved. While effective in binding freshwater algae, a high salinity environment results in poor alga-resin interaction. Susceptibility to resin fouling appears to be related to the type of algae used. Finally, different resin surface groups may be used in order to increase overall biomass binding capacity. Further development of polymer materials is in order for improving biodiesel production economics and advancing the adoption of algae-based biofuel.

Chapter 6: Resins that reversibly bind algae for dewatering and concentration

6.0. ABSTRACT

Algae have great potential to address a number of important needs such as water remediation and as a feedstock for biofuel and biochemicals. At present, harvesting or removing algae is expensive, especially in terms of the cost of making biofuel. The expense of harvesting algae arises from the cost of pumping and processing large amounts of water. In an effort to explore less expensive methods for harvesting we have developed resins that bind and release algae as a function of pH. Here we developed a series of weak anion exchange resins incorporating various weak bases with different pKa values. The best of these resins bind about 10% of their weight in algae, show 100% reversibility and reusability, and the ability to concentrate algae from dilute suspension to 30 g/L. Furthermore, they have the potential for desalting algae grown in high salt media. These resins provide a low cost method for harvesting algae without pumping water or introducing large amounts of chemicals into the feedwater, harvested algae, or the environment.

6.1. INTRODUCTION

Algae have the potential to address current and future energy needs as feedstock for biofuels (Chisti 2007). High oil content and biomass production rates (Li et al. 2008), as well as higher photosynthetic efficiencies than terrestrial plants (Li et al. 2008), make algae ideal for commercial-scale biofuel production. Algae consume carbon dioxide which can reduce greenhouse gas emissions compared to petroleum and energy cost balances can be improved when using flue gas as a carbon source (Li et al. 2008).

Furthermore, algae can be cultivated in brackish water unsuitable for agricultural crops, and be used to remediate wastewater (Munoz and Guieysse 2006). In addition to fuel, profitable co-products can be generated, such as animal feed, biopolymers, and agricultural fertilizers (Bringezu et al. 2009). Algae are a sustainable source of high-value biochemicals (Gavrilescu and Chisti 2005); for example, docosahexaenoic acid (DHA), an essential omega-3 fatty acid important to infant development and cardiovascular health (Pyle et al. 2008), is synthesized and concentrated in algae (Harwood and Guschina 2009).

One of the current barriers to using algae for biofuel is the cost of growing and harvesting algae from dilute suspension. Most algae used for biofuel are small, 2-3 micrometers in diameter, and are difficult to separate from water by filtration or tank settling (Uduman et al. 2010). Furthermore, autotrophic algal growth density is limited by light perfusion. While lab-scale studies report biomass density in the magnitude of grams per liter media (Banerjee et al. 2002), large-scale cultivation, such as in a raceway pond, often produce biomass concentrations of less than 0.5 g/L (Chisti 2007). As a consequence, large volumes of water must be processed in order to generate the amount of biomass needed. For example, to recover just one kilogram of algal dry mass from an algal culture of 0.4 g/L density (a typical biomass density observed in our photobioreactors), at least 2500 liters (660 gallons) of water must be processed. Previous studies have shown that biomass recovery contributes 20-30% of the total production costs (Grima et al. 2003). Thus, the cost of water removal from algal biomass poses a significant obstacle to the expansion of algae-based bioenergy (Uduman et al. 2010).

Current techniques used on the commercial production scale have proven inadequate, requiring considerable time or energy expenditure to harvest the algal biomass. Mechanical separation methods such as centrifugation produce the highest

biomass yields, but also have high equipment and energy costs (Grima et al. 2003; Olaizola 2003). Flocculation, the induction of algal cell aggregation, can be achieved by either charge neutralization or addition of charged polymers that coordinate the charged algal surfaces. Although operating cost can be low, depending on the flocculant, it involves continual addition of chemicals to large volumes of water. Some of these treatments introduce unwanted salts or metal ions whereas others may have long residence times and, in any case, can affect the output water (Li et al. 2008). Experimental harvesting techniques, such as ultrasound-induced aggregation, have been effectively demonstrated at the laboratory scale, but have been projected to be more expensive then current centrifugation techniques (Bosma et al. 2003).

Problems with the current methods inspired us to design, develop, and test reusable resins for concentrating algae out of suspension. The strategy takes advantage of the negative surface charge on algae, which bind to weak anion exchange resins as a function of pH. While simple in principle, the performance of commercial resins was disappointing due to non-specific or non–pH dependent binding, as well as poor binding capacity. Here we studied the binding capacity and algal release as a function of resin chemistry. Through several generations of synthesis and analysis we were able to progressively improve both binding capacity and release properties of the resins. The best of these resins can bind ten percent of their weight in algae with essentially 100% reversibility and the ability to concentrate algae 100-fold from dilute suspension.

6.2. MATERIALS AND METHODS

Amberlite® IRA-67, Aminomethyl Chemmatrix®, Celite 500, Dowex® Marathon™ WBA free base, Dowex® Retardion 11A8, Levatit® MP-62 free base,

poly(4-vinylpyridine hydrochloride), polymer-bound aniline, Sephadex™ G-25, Stratospheres™ PL-DETA, Stratospheres™ PL-DIPAM, Stratospheres™ PL-PPZ, and polymer-bound TETA were obtained from Sigma (St. Louis, MO). Amberlite® CG-400 was obtained from Mallinickrodt (St. Louis, MO). DEAE Sephadex™ was obtained from Pharmacia Biotech (Sweden). Silica gel was obtained from EMD Chemicals (Gibbstown, NJ). Dimethylaminoethyl methacrylate (DMA), divinylbenzene (DVB), ethylene glycol dimethacrylate (EGDMA), methyl methacrylate (MMA), styrene (S), vinyl 2-imidazole (IM), vinyl 4-pyridine (PYR), 2-hydroxyethyl methacrylate (HEMA), and azobisisobutyronitrile (AIBN) were obtained from Sigma.

Neochloris oleoabundans (UTEX LB 1185) was obtained from The University of Texas at Austin Culture Collection of Algae (Austin, Texas). KAS 603, a saltwater species of *Chlorella*, was obtained from Kuehnle Agro Systems (Hawaii).

6.2.1. Algal cultivation and harvest

Neochloris oleoabundans was cultivated in freshwater (approximately 5 psu) Bold 3N (B3N) medium (Brown and Bold 1964), and *Chlorella sp*. was cultivated in saltwater (approximately 35 psu) f/2 medium (Jeffrey and LeRoi 1997). Both cultures were grown at room temperature (23 °C) under cool white fluorescent lights on a 12 h:12 h, light:dark photoperiod in airlift photobioreactors aerated with ambient air using aquarium pumps. Immediately prior to testing, algae were concentrated by centrifugation (Sorvall Legend XTR) at 8000 RPM for 10 min. The supernatant was decanted and the pellet resuspended in B3N medium at concentration of 0.2 g/L. Media was tested at freshwater salinity level (5 psu) and pH 6.5, unless otherwise noted.

6.2.2. Commercial resin testing

Commercially available resins were evaluated for the ability to bind and release algae. For each assay, 100 mg of washed resin was loosely packed into a polyethylene column. 100 mL of algal suspension at 0.2 g/L was loaded into the column until the resin reached binding saturation. The optical density of the algal suspension at 680 nm (OD_{680}) loaded onto the column and recovered in the eluate was measured for quantifying binding capacity. The resin was rinsed with distilled water, and then eluted with 10 mM sodium phosphate at pH 12 to release algae.

6.2.3. Resin synthesis

Monomers were combined in ratios based on weight as indicated in Table 6.1. Crosslinkers, EGDMA or DVB, were used to form polymethacrylate (PMA) and polystyrene-based (PS) resins, respectively. Functional monomers tested include DMA, IM, PYR, and HEMA. Remainder of resin weight was occupied by spacer monomers: MMA for PMA resins, or S for PS resins. An equivalent volume of solvent was added to the combined monomers, consisting of 50% toluene and 50% acetic acid:water (1:30 v/v). Porosity of the crosslinker network was held constant by keeping toluene at the same weight percentage for all resins. Resin synthesis was carried out in round bottom flasks fitted with an argon bubbler and heated to 60 °C with constant stirring. Polymerization was initiated by addition of one mol percent AIBN and polymerization continued until the mixture formed a brittle solid. The polymer was then dried in 55 °C oven for 12 h, scraped from the flask and ground by mortar and pestle. The crushed resin was then sized between 35 and 170 size stainless steel meshes to obtain macroporous beads of approximately 100-500 μm diameter.

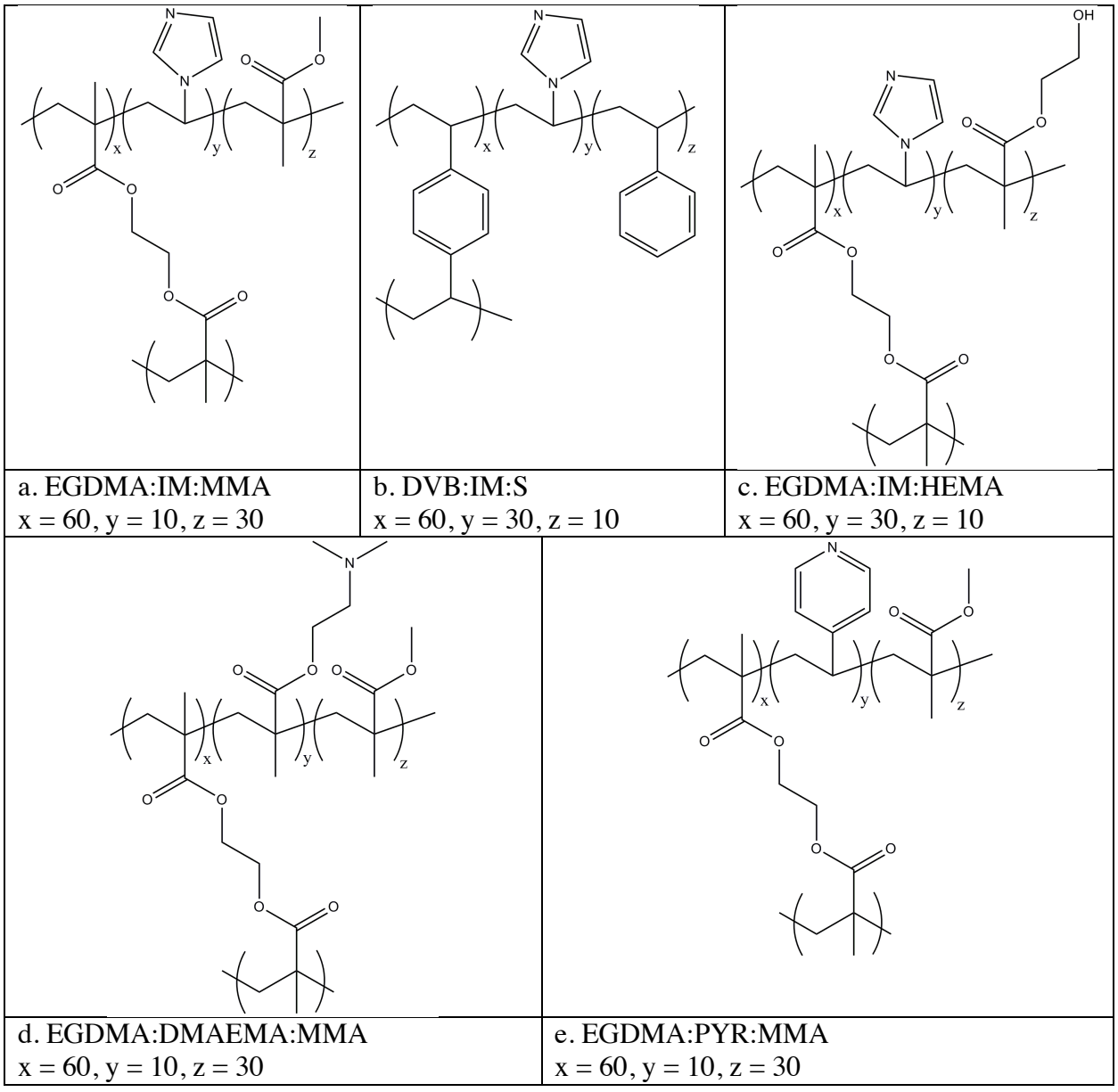


Table 6.1: Structures of pH-dependent reversible alga-binding resins.

6.2.4. Determination of algal dry cell weight

Routine determination of algal dry cell weight (DCW), was obtained by measuring OD_{680} using a Shimadzu spectrophotometer. To convert OD_{680} to DCW, the OD_{680} was recorded for an algal dilution series and then the contents of each cuvette were collected onto pre-weighed cellulose acetate membranes (Pall Co., Port Washington, NY). The membranes were then dried in a vacuum oven (15 in. Hg., 60 °C) for 12 h and then weighed to give the DCW for a given OD_{680}. To avoid optical filtering effects, algal suspensions were diluted if needed, to keep the OD_{680} under 1.8.

6.2.5. Algal binding and release assay

To test algal binding capacity and release characteristics of newly synthesized resins, 1 g of resin was added to 100 ml of 0.2 g/L algal suspension in a 250 ml flask. The suspension was then gently agitated on an orbital shaker (VWR, 125 RPM) for 15 min. Subsequently, the suspension was filtered through #170 stainless steel mesh to isolate the resin and the OD_{680} of unbound algae was measured. The difference in OD_{680} obtained before and after resin binding was used to determine how much algae was bound to the resin. The resin was then transferred to a second flask containing 100 mL of 10 mM sodium phosphate buffer at pH 12 and the mixture was again agitated for 1 h. The resin was then removed by filtration through the steel mesh and OD_{680} of desorbed algae was recorded. To determine the pH dependence of algae unbinding, the same procedures were carried out except that algae were eluted using 100 mL of 10 mM sodium phosphate buffer adjusted to have a pH over the range of 8 – 12.

To determine how well resins could concentrate algae, the procedures were the same as for the previous elution studies except that 1 g of resin was added to 500 mL of

0.2 g/L algal suspension in a 1 L flask. Algae were then eluted off the resin with different volumes (500, 50, 25 or 10 ml) of 10 mM sodium phosphate buffer at pH 12.

Experiments were based on samples run in triplicate at the same time. Mean DCW bound and released per gram resin was reported, with brackets indicating standard error of the mean.

6.3. RESULTS

The algal binding and release assay is shown in Figure 6.1. For better illustration, the figure shows binding and release of algae from a resin-packed column. Algae was added to the column and bound to the resin (6.1b); the clear growth media was recovered for immediate culture reuse. Addition of basic buffer immediately began to release algae from the resin, and the desorbed algae were eluted with the buffer from the column (6.1c). Though trace chlorophyll pigments from some algal species would stain the resin, this did not diminish subsequent algal binding. The column photographed was reused and regenerated in this manner three consecutive times.

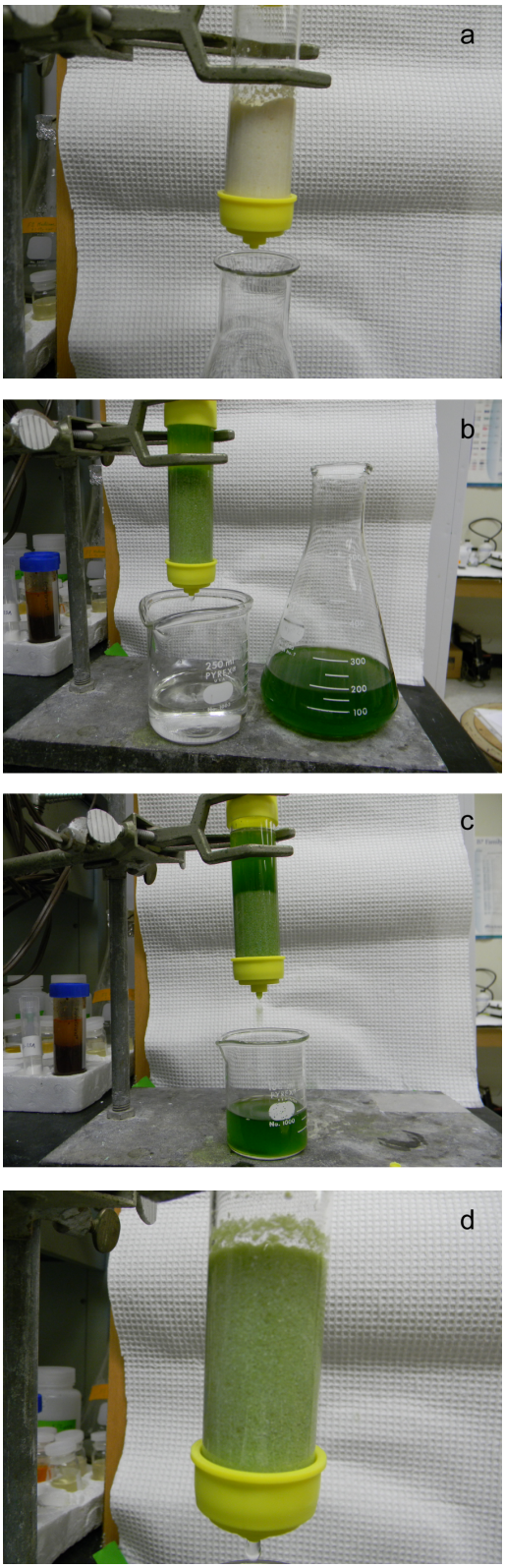


Figure 6.1: Algal binding and release assay.

Resin column (a) is loaded with algal suspension; as algae is bound to the resin, the clear growth media is recovered (b). After binding saturation, regeneration buffer is used to release algae from the resin (c). The resin is cleared and reused for another load of algae (d).

In order to quantify masses of algae bound and desorbed from the resin by optical measurement, chlorophyll optical density calibrations were determined for both algal species. The calibration fits were linear over the optical density range tested (R^2_{Neo} = 0.997, R^2_{Chl} = 0.999) (Figure 6.2). DCW obtained by optical measurement was validated against gravimetric measurement of original algal suspension and released algae.

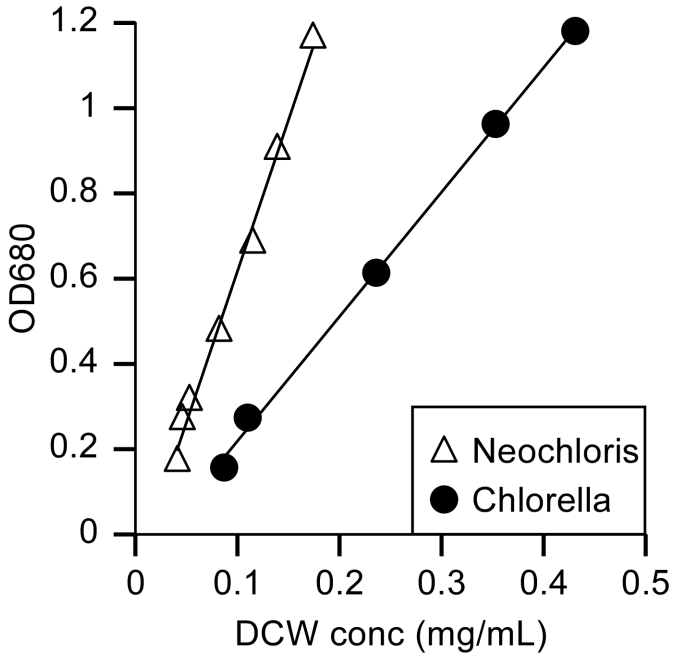


Figure 6.2: Algal biomass quantification.

Chlorophyll optical density calibration standard at 680 nm as a function of *Neochloris* and *Chlorella* DCW concentration.

Previous studies had shown that strong anion exchange resins could bind algae. However, once algae bound to the resin, they could only be eluted by extreme conditions. This could be due to the fixed charge on the resin but it might also be due to other interactions between the resin and the algal surface. For example, the results shown in Table 6.2 indicate that Amberlite CG-400 and IRA 67, DEAE Sephadex, polymer-bound pyridine, and aminomethyl Chemmatrix were able to bind algae, but algae could not be removed by base. On the other hand, a number of resins and chromatography materials including Dowex Marathon WBA and Retardion 11A8, Levatit MP-62, Celite, silica gel, and Sephadex did not bind to algae even though some of them are anion exchange resins. A few of the resins, TETA resin, Stratospheres resins PL-PPZ, PL-DETA, and PL-DIPAM, and polymer-bound aniline, did show some ability to bind and release algae.

Based on preliminary results with commercial resins, we concluded that weak anion exchange resins showed the most promise but the resin matrix was also important. Furthermore, we suspected that resin-bound weak bases with pKa values near 7 would be easier to elute than those with high pKa values. With that in mind we generated a series of resins incorporating one of three different functional groups, dimethylamine (DMA), imidazole (IM) and pyridine (PYR) (Table 6.1).

Resin	Functionalization	Algal binding	Algal release
Polymer-bound TETA	triethylene tetramine	+	+
Stratospheres PL-PPZ	piperazine	+	+
Stratospheres PL-DETA	diethyethylene triamine	+	+
Stratospheres PL-DIPAM	diisopropyl ethylamine	+	+
Polymer-bound aniline	aniline	+	+
Amberlite	quaternary ammonium	+	-
DEAE sephadex	diethylamine	+	-
Amberlite IRA 67	amine	+	-
Poly(4-vinylpyridine HCl)	pyridine	+	-
Aminomethyl Chemmatrix	amine	+	-
Dowex Marathon WBA	amine	-	n/a
Dowex Retardion 11A8	paired anion and cation sites	-	n/a
Levatit MP-62	weak base	-	n/a
Celite 500	none	-	n/a
Silica gel	none	-	n/a
Sephadex G-25	none	-	n/a

Table 6.2: Commercial resins evaluated and their surface functional groups.

The binding capacity and adsorption reversibility was tested for DMA, IM, and PYR incorporated at 10% wt into PMA resin, Figure 6.3. Higher binding capacity was observed for DMA and IM resins, with 34.2 and 25.3 mg of algae bound per gram of resin, respectively, while PYR was less at 13.6 mg. After algae release at pH 12, 23.2 (68% of bound algae released), 23.3 (92%), and 16.0 mg algae per g resin (100%) was unbound from DMA, IM, and PYR, respectively. Given the relatively poor binding capacity of the PYR resin at pH 6.5, further studies focused on the DMA and IM resins.

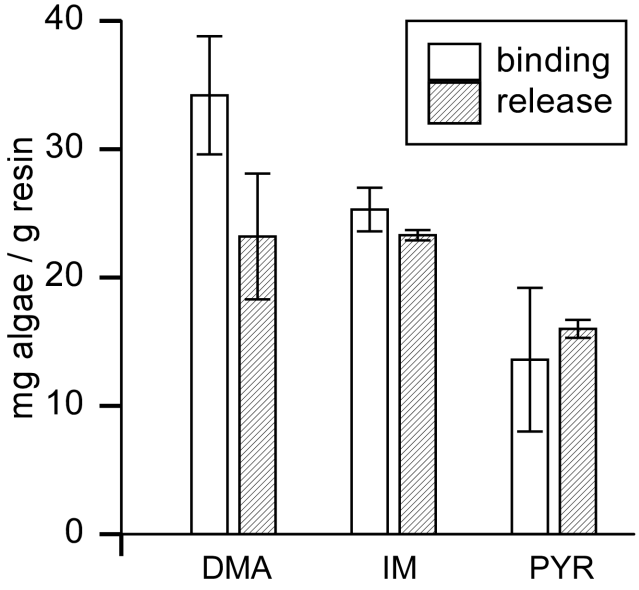


Figure 6.3: Algal binding by resin functional group.

Comparison of weight of algae bound and released per gram resin for dimethylamine (DMA), imidazole (IM), and pyridine (PYR)-functionalized resins. Bars represent average of three replicates, brackets indicate standard error of the mean.

The initial binding and release data showed that binding capacity correlated positively with increasing pKa of the weak base component while reversibility correlated inversely with pKa. Binding reversibility was further examined for DMA and IM resins by desorbing algae over a range of pH values. The proportion of algae released at each pH is shown in Figure 6.4. At lower pH 8 and 9, algae remain largely fixed to both resins. For DMA resin, 13.1% and 28.1% of bound algae was released at pH 8 and 9, respectively, while for IM resin, 22.5% and 32.1%. At pH 10, DMA released 33.8% of algae back into suspension, while IM released 70.5%. When pH was increased to 11 and

12, algal binding was mostly reversed, with DMA resin releasing 86.5% and 100% of algae from the resin, and IM resin releasing 77.2% and 92%.

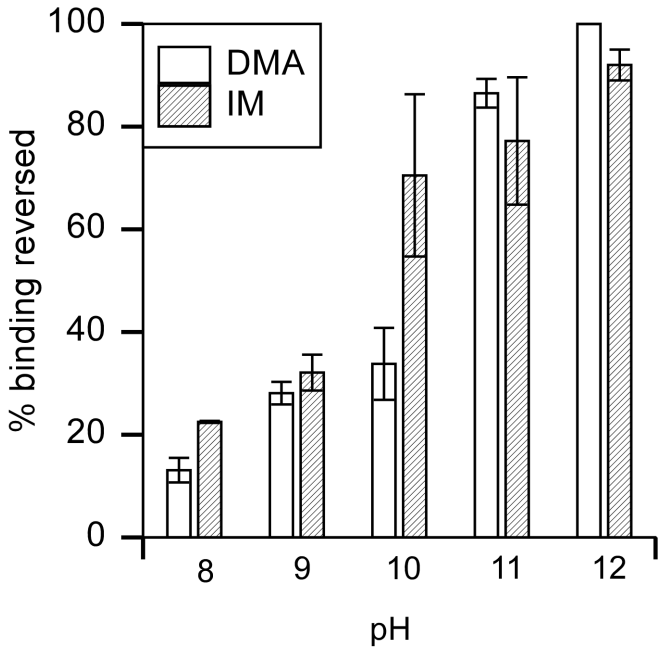


Figure 6.4: Removal of algae over range of pH.

Binding reversibility of DMA and IM resin in regeneration buffers, pH 8 – 12. Bars represent average of three replicates, brackets indicate standard error of the mean.

Having observed that the IM functional group was effective at both binding and releasing algae, the effect of resin crosslinker was examined. Initial results showed that IM incorporated into PS resin at 60:10:30 w/w/w ratio of DVB:IM:S bound algae poorly. However when the ratio of IM was increased to 60:30:10 w/w/w DVB:IM:S, binding was comparable to that of the methacrylate resin. We then compared IM incorporated into PMA resin (EGDMA:IM:MMA, 60:10:30 w/w/w) to the PS resin (DVB:IM:S, 60:30:10 w/w/w). For these comparisons, we assayed the binding of *Neochloris* in freshwater B3N

medium and *Chlorella* in freshwater, brackish, and saltwater f/2 media (7, 15, and 32 psu). The results show that PMA resin (Figure 6.5a) had the highest binding capacity for *Neochloris* (34.2 mg algae per g resin) whereas binding for Chlorella was considerably lower in fresh water (12.8 mg/g) and decreased even further in brackish (10.9 mg/g) and sea water 5.1 mg/g). The PS resin (Figure 6.5b) was found to have a higher binding capacity for *Chlorella* in freshwater medium (41.2 mg/g) than *Neochloris* (23.5 mg/g). As with the PMA resin, *Chlorella* binding capacity was reduced at medium and high salinity (14.6 mg/g, 12.5 mg/g).

The data showed that binding of algae to some of these resins was not 100% reversible and that there was some binding that was not simply due to the charge on the resin. To investigate this further, other resin compositions were tested. Interestingly, we found that inclusion of HEMA in the resin improved both binding capacity and reversibility. Initial studies showed that EGDMA:IM:HEMA, 60:30:10 w/w/w had a binding capacity of 91.0 mg /g resin and essentially 100% reversibility with *Chlorella* in freshwater medium. Removal of algae was tested with decreasing volumes of releasing buffer in order to determine if the resins could yield a more concentrated algae solution after binding. Figure 6.6 shows that after binding, comparable amount of algae can be released into smaller buffer volumes. Eluting in this manner resulted in concentrated algae with densities of 2.19 g/L (8.8X concentrated), 3.03 g/L (12.4X), 19.0 g/L (57.3X) and 29.6 g/L (110X). At higher concentrations, it became increasingly more difficult to completely release algae into smaller volumes; 100% of the algae were recovered in 8.8X concentration, and 83%, 70%, and 47% of algae was recovered in the eluate at 12.4X, 57.3X, and 110X, respectively. Fortunately, additional washing was able to release the remaining algae, indicating that the remaining algae was entrained in the beads but not necessarily bound to them.

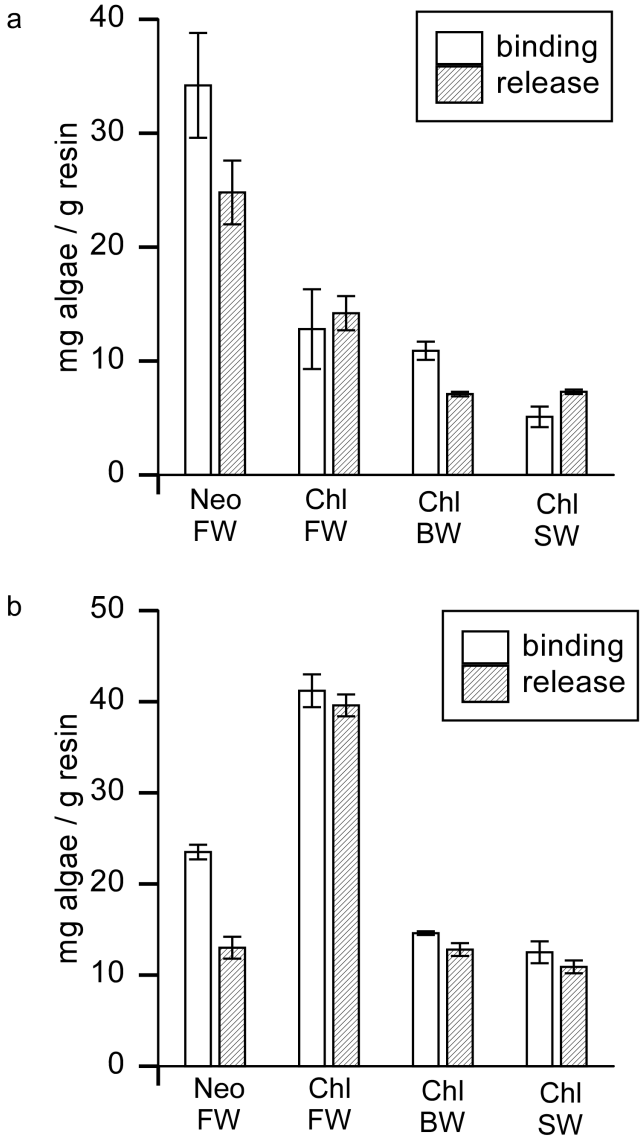


Figure 6.5: Algal binding in different growth media.

Comparison of weight of algae bound and released per gram resin for IM resins using *Neochloris* in B3N medium, and *Chlorella* in freshwater, brackish, and saltwater salinity f/2 medium using PMA resin (a), and PS resin (b).

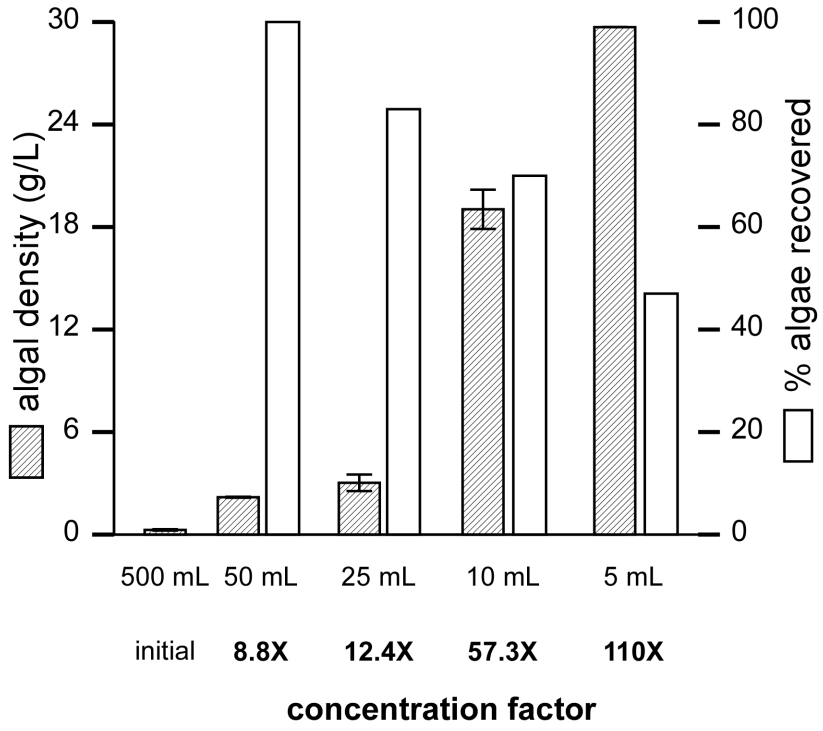


Figure 6.6: Concentration of algae.

Concentration of algae recovered from high-capacity resin (EGDMA:IM:HEMA, 60:30:10 w/w/w) in limiting volumes of regeneration buffers, producing algae solution at 1.1, 8.8, 12.4, and 57.3-fold concentration of original suspension.

6.4. DISCUSSION

Our study was developed after observing that while commercial anion exchange resins were effective at binding algae out of suspension, binding was not easily reversed. The commercial resins that we tested are marketed for a broad array of applications, including chemical purification, water treatment, and polymer-supported synthesis, and were selected based on amine or base surface functionality that could potentially interact with the algae. As a control, materials without surface functionality, such as silica, were tested, and it was confirmed that no algal binding occurred. In addition, some resins with weakly anionic groups, such as Dowex Marathon WBA and Retardion 11A8, were unable to bind algae at all. Strongly basic resins, such as Amberlite CG-400, were able to bind algae out of solution, binding approximately 30 mg of algae per g of resin, but binding could not be reversed by elution with buffers up to pH 12. Resins for polymer-supported chemistry, such as Stratospheres PL-PPZ, PL-DETA, and PL-DIPAM, were successful at both binding and releasing the algae. However, algal binding capacity was very low, less than 10 mg of algae per g of resin. Also, since these resins are manufactured for niche market, they are only available for purchase at small quantities for high cost. However, the results provided some initial guidelines for subsequent resin synthesis.

The resins we synthesized had a high crosslinker density, and proved to be non-swelling, hard resins suitable for testing. As the constituent ratios in the simple co-polymer resins used in these studies can be rapidly altered and additional resins rapidly synthesized, this permitted resins with different compositions to be rapidly screened. This variation allowed us to study binding behavior that was based on ionic functional group.

Initially, assays were conducted in upright resin-filled columns. This proved to be problematic, in that the resin could pack unevenly, leading to channeling within the column. This approach resulted in an uneven column flow rate, a variable affecting resin residence time, and the binding and release behaviors. In addition, the column frit rapidly accumulated a layer of desorbed algae that further impeded flow, especially during concentration experiments when a minimum amount of buffer was used for elution. The algae binding and release experiments conducted in flasks resulted in more reproducible adsorption and desorption results.

Optical chlorophyll measurements were used to expedite the resin screening process, and were used as an indication of viable cells with higher specificity than measuring algal DCW using a gravimetric method. The optical chlorophyll measurements and the gravimetric measurements performed on a sample of the released algae were found to be comparable.

The polymer resin's ionic functional group was expected to be the main alga-interacting component, similar to anion exchange resin. Algae are unicellular plants with cell walls composed of biopolymers, which have carboxyl groups that are ionized at the pH of the media (Onyancha et al. 2008). Since these carboxyl groups are common to all algae, the polymer resin harvest technique can be applied to all algal cultures.

With greater difference between the media pH and the functional group pKa, an improvement of binding capacity was observed. DMA, with a pKa of 10.2, consistently demonstrated high binding capacity, and IM, with pKa 7.7, also bound algae reasonably well. The PYR resin with pyridine functional groups having a pKa of approximately 5.5 was also the weakest binding resin. This is to be expected since the pH of media is above the pKa and only a fraction of the pyridines should be ionized.

From the various tests, IM was eventually favored over DMA because of its better binding reversibility at lower pH. Because of IM's lower pKa value, it could be reverted to a non-ionized form with less of a pH shift than DMA. The fact that the majority of algae was released at pH 10 for IM resin while for DMA required at least pH 11 in to release most of the algae is significant in terms of the practical application. While a shift of one pH order seems small on a benchtop scale, in an industrial process it would require ten-fold more base.

Interestingly, while control PMA and PS resins without ionic functional groups did not exhibit algal binding behavior, we found that the choice between PMA and PS did subtly affect the overall binding capacity of the functionalized resins. The observed difference in binding capacity may be related to the hydrophilicity of the crosslinker and spacer groups. The PMA resin consists of hydrophilic monomers that make the resin more wettable, facilitating the interaction between the ionic functional groups and the algal surface. When the MMA spacer was replaced by HEMA, the hydroxyethyl group in the latter resin increased the hydrophilicity of the resin, and the binding capacity also increased. In contrast, the PS resin consists of hydrophobic monomers that weakly repel the algae. It was possible to obtain PS resins that showed similar binding capacities to the PMA resin but it required three times higher concentrations of the IM monomer.

Binding reversibility depended not only on the ionic functional group, but also on the resin matrix. We were able to synthesize resins that bound reversibility, even though algae could not be released from commercial resins with similar functional groups, such as DEAE Sephadex and poly(4-vinylpyridine hydrochloride). In our own resins, irreversible binding between *Neochloris* and PS resin was observed, suggesting that some algal species have hydrophobic surface groups that interact strongly with hydrophobic polymers and can lead to resin fouling.

While the binding and release of algae from the resin was demonstrated in equilibrium conditions, an important function of algae harvest is the removal of water from the biomass. Thus, an experiment was conducted to determine if algae could be completely recovered off the resin using smaller volumes of buffer solution. 57-fold concentration was easily obtained with our assay, yielding a slurry of 19 g/L biomass density. Alternatively, we found that batches of algae could be bound to the resin, and then eluted repeatedly with the same elution buffer, also yielding an algal concentrate. The experiment demonstrated that the saturation of the basic solution was not the main limiting factor in algae concentration to consider.

We achieved our goal of concentrating the algae over 100-fold by eluting the resin with just enough basic buffer to recover a concentrated paste of 29.7 g/L. We were able to remove 47% of the bound algae in the elution step, though additional rinsing with distilled water was enough to recover the remainder of the algae. The technical issue involved the resin beads, which was increasingly difficult to filter off of the turgid algal paste as the concentration factor increased, and not the reversibility of the resin, which was achieved even with small buffer volumes.

This study demonstrated that functionalized resin could be used to bind and concentrate algae out of a dilute suspension. The high binding capacity resin tested in the concentration experiments, with capacity approximately 0.1 gram of algae per gram resin, was used as a specification benchmark for projecting the industrial application of binding polymer. For example, the high surface area of the resin beads could be matched by a resin-coated bristle surface. A schematic for such an implementation is shown in Figure 6.7. A conveyor belt, 1 meter in depth and 7.5 meters in length, covered with bristles that are 1 millimeter in diameter and 10 centimeters in length, could process 7500 L of water (1981 gal) and recover 3 kg of algae from a typical pond with algal density of 0.4 g/L.

Such a belt could be placed within current raceway ponds, which already require paddles for culture circulation. To remove the algae, the belt could either be physically removed and cleared, or could cycle through a separate tank for algal concentration.

While apparently similar to chemical flocculation, the polymer harvesting method offers specific advantages. First, the polymers quickly adsorb algae in the neutral pH range of the medium. Flocculation by pH shift requires addition of sufficient sodium hydroxide or lime to raise the pH of the entire body of water to be harvested. Indeed, enough alkali must be added to overcome the intrinsic buffering power of the growth media and induce supersaturated conditions where the precipitate comes out of solution quickly. With resins, a much smaller volume of elution buffer is needed.

Furthermore, although our resins are less efficient in seawater they still will bind algae under high salt conditions, while all commercial resins tested were ineffective at binding in salt water. Since the volume of elution buffer used is considerably smaller than the original dilute growth suspension, only a small amount of alkali is used to release the algae. The eluted algae will effectively be desalted. This is important when considering the use of algae grown in saltwater for feedstock or fertilizer.

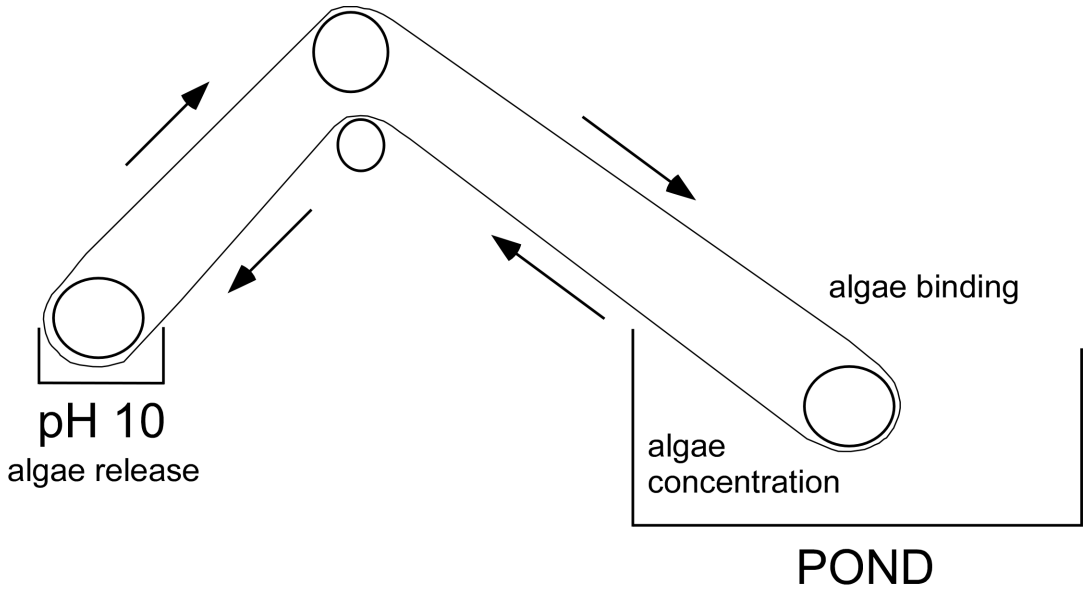


Figure 6.7: Commercial application of harvesting resins.

Schematic for algal harvest by reversibly-binding resins in industrial application. The resin is applied as a high surface area substrate over a rotating platform, such as a conveyor belt.

6.5. CONCLUSION

The need to decrease fossil fuel dependence is an immediate technological concern. In order to compete with low-value petroleum products, algae biomass, more so than other high-value energy feedstocks, must be produced with low cost and highly efficient methods. This study demonstrates the application of a simple principle to harvest algae with less energy input than mechanical methods. It is an improvement over flocculation techniques in that it is reversible, nontoxic, and that the binding substrate is recoverable and reusable.

Chapter 7: Conclusion

Due to its high energy density, abundance, and the relative ease with which it can be extracted from the earth, fossil fuels have been the energy standard since the nineteenth century. Relatively little research attention has been given to alternate energy sources until recently. Prompted by environmental and geopolitical concerns, as well as the knowledge that petroleum fuel reserves are becoming rapidly depleted, the demand for cheap, convenient, and ecologically-sound energy continues to rise.

Algae are the most sustainable biofuel option. One of the main concerns regarding biofuel production is that it will draw from the food supply; fortunately, algae are not agricultural crops. Algae are capable of prodigious growth and high oil content. The carbon fixation and wastewater remediation activities by algae can address increasing greenhouse gases and water pollution, respectively, which are both important environmental concerns. However, there are obstacles to widespread adoption of algae as a fuel source. Algae are expensive to cultivate, and large-scale growth conditions diminish biomass and oil productivities. Traditional harvesting practices cannot be applied to algae since they are cultivated in large volumes of water, and the pumping and transport of so much water increases production costs. Water in the biomass inhibits both oil extraction and transesterification, but completely drying a large biomass is prohibitively expensive. Current oil extraction methods are also costly because of the difficulty in separating oil from the residual biomass. Because of these obstacles, production of a commercial biofuel has been difficult to achieve.

This dissertation describes the application of low-cost polymers that improve the economics of biofuel production from algae. Three processes in biofuel production were identified as areas for improvement: oil extraction, biodiesel synthesis, and biomass

harvest. All the resins developed were based on inexpensive styrene and acrylic scaffolds that could be synthesized on the industrial scale. While the principles have been explored in this dissertation, more work must be conducted before polymer-based methods can be applied to commercial biofuel production.

Hydrophobic resins were developed that act as solid phase extractors and selectively bind algal oil from suspension (Chapter 4). Polystyrene and polymethacrylate resins were functionalized with hydrophobic and hydrophilic surface groups, or imprinted with triacylglycerol recognition sites. Lysed algal suspension was passed through a resin column to accumulate oil, while the residual biomass was recovered without solvent contamination. A high-yield, selective algal oil separating resin was not identified during the course of the study. Instead, the most promising finding was that treatment with dilute ethanol was sufficient to disrupt the algal cells and allow efficient oil binding; future work should focus on resins that can simultaneously disrupt cells and accumulate oil.

It was observed that materials such as anion exchange resins were able to recover algae out of dilute suspension (Chapter 5). The fixed charge of surface ammonium groups bound to negatively-charged algal surfaces. Collection of algae onto an anion exchange column resulted in a dewatered biomass for direct biodiesel synthesis. Elution with transesterification reagent served the dual purpose of generating biodiesel and regenerating the resin. The quantity of FAME generated by resin-bound algae was comparable to that of a dried algal pellet. Transesterification with the dried pellet used heat for both drying and FAME synthesis; however the resin-bound algae produced similar results with no heat inputs. The binding capacity and resin reusability was also compared between two algal species and found to be species-specific.

Since removal of algae from the anion exchange resin resulted in destruction of the cells, new polymers were designed that could bind algae reversibly with high binding

capacity (Chapter 6). Polystyrene and polymethacrylate resins were functionalized with basic groups that were ionized at neutral pH. Inclusion of the hydrophilic monomers further improved binding capacity. Algae were harvested without any treatment of the growth media, and released as a concentrated solution in a resin-regenerating solution. A resin that bound 10% of its weight in algae and concentrate biomass 50-fold was developed in the study. The resin could also be regenerated to nearly 100% of its original binding capacity.

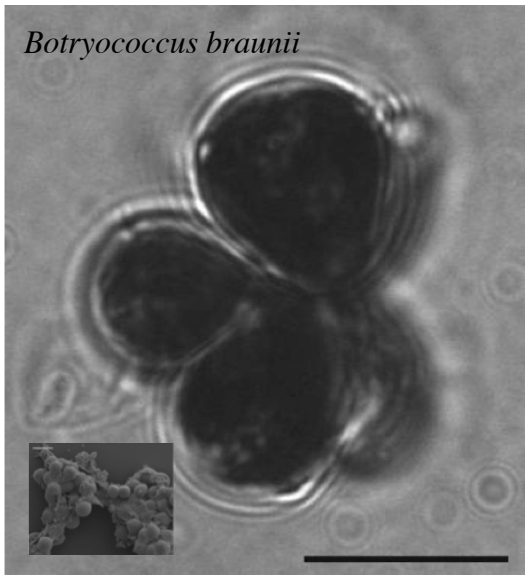
For scaling up the biomass harvesting resins, the primary concerns are to bind a large quantity of algae using the least amount of material, and to easily regenerate the material for reuse. The presented research was based on rigid, nonswelling resin beads with high surface area; a commercial harvesting system must either have a similarly high surface area for binding, or an increased binding capacity. Additionally, the polymer was easily regenerated by treatment with high pH media. In the future, process engineering aspects such as binding and removal kinetics, the maximum binding distance allowable between resin and algal cell, recycling of the resin-regenerating solution, and gradual polymer fouling over continuous use must be examined.

There is still no definite answer as to which energy mode will answer current and future demand for fuel. However, algae-based biofuel has already reached several important milestones with regards to its future adoption: algae are cultivated at the industrial-scale (hundreds of tons per year) by several companies; there are currently over 100 companies developing algae-based biofuel technologies, and many that are launching pilot biofuel plants; stock shares of several algal product companies (*e.g.*, OriginOil, PetroAlgae, PetroSun, Solazyme) are traded publicly; government initiatives continue to promote research on algae, and the military has targeted algae as a renewable source of jet fuel. Given the current climate of interest, it appears that algae are the way to

becoming a commercially-viable biofuel. The predicted time to adoption ranges anywhere between 10 to 50 years time. Supplanting fossil fuel use with alternative energy sources such as algae-based biofuel is undoubtedly an important and critical engineering goal.

Appendix: Taxonomy of featured algae

The algal species named in the dissertation are sorted by phylum and **class**. DIC and SEM micrographs (insets) by the author. DIC bars (black) are 10 micrometers and SEM bars (white) are 2 micrometers.



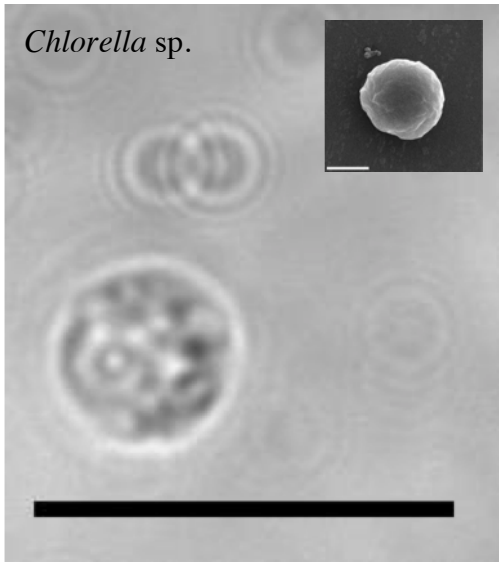
Chlorophyta (green algae)

Chlorophyceae

Ankistrodesmus falcatus – high lipid content (24% of dry wt.) in enriched nutrient conditions (Huber et al. 2006); used in phycoremediation (Munoz and Guieysse 2006).

Botryococcus braunii – colonial algae with high secreted hydrocarbon content (>60% of dry wt.); slow growth rate (Metzger and Largeau 2005).

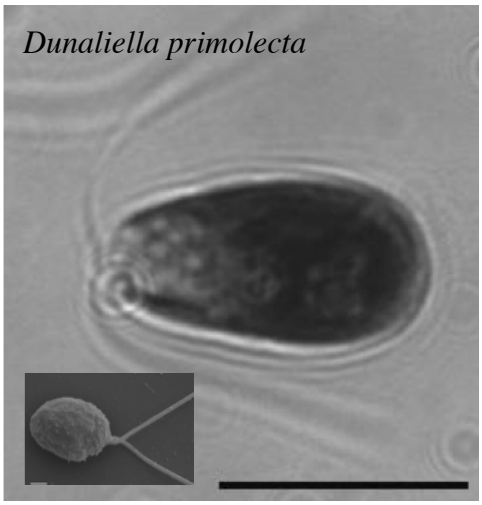
Chlamydomonas reinhardtii – model alga for genetic manipulation studies of lipid synthesis (Dunahay et al. 1996); not known as a neutral lipid producer.



Chlorella protothecoides – grown heterotrophically for high lipid content (55% of dry wt.) compared to autotrophic growth (14% of dry wt.) (Miao and Wu 2006).

Chlorella sp. – grown as food product (Benemann et al. 1982); highly productive (Illman et al. 2000) and robust species, has been

grown with flue gas (Doucha et al. 2005).



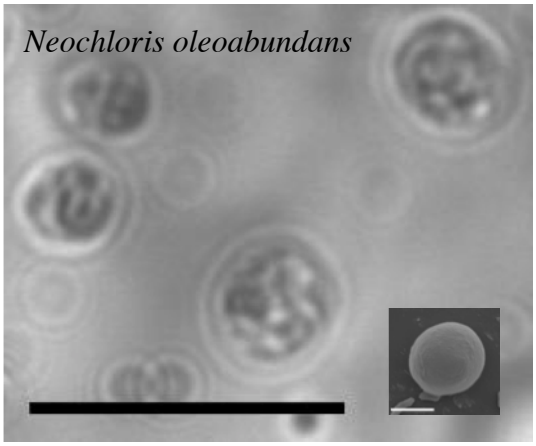
Dunaliella salina – high lipid content (25% of dry wt.) in enriched nutrient conditions (Huber et al. 2006); grown for *beta*-carotene (Hejazi et al. 2004); thrives in high salt media (Pulz and Gross 2004).

Dunaliella sp. – species including *D. tertiolecta* and *D. parva* have high reported glycerolipid content and compatibility with high salt environments (Harwood and Guschina 2009; Tran et al. 2010).

Haematococcus pluvialis – cultivated for anti-oxidant astaxanthin (Miao et al. 2006).

Neochloris oleoabundans – high lipid content (80% of dry wt.) when nitrogen-deprived (Tornabene et al. 1983).

Scenedesmus obliquus – freshwater alga (Rodolfi et al. 2009); high lipid content (21-42% of dry wt.) (Salim et al. 2010).



Scenedesmus sp. – freshwater alga (Rodolfi et al. 2009); high lipid content (20% of dry wt.) (Rodolfi et al. 2009), used in wastewater remediation (Munoz and Guieysse 2006).

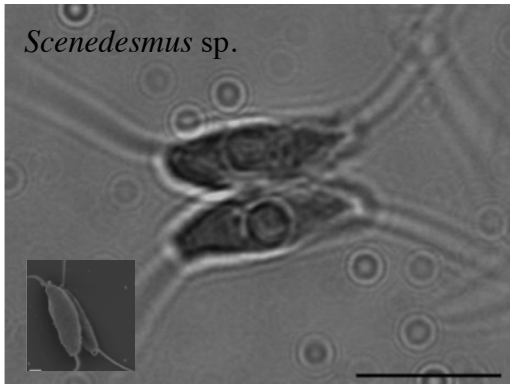
Tetraselmis suecica – marine alga (Rodolfi et al. 2009); high lipid content (18-26% of dry wt.) (Salim et al. 2010).

Volvox carteri – model alga for genetic manipulation studies of lipid synthesis (Beer et al. 2009); not known as a neutral lipid producer.

Dinophyta (dinoflagellates)

Dinophyceae

Crypthecodinium cohnii – accumulates high amounts of DHA (20% of dry wt.) (Harwood and Guschina 2009).



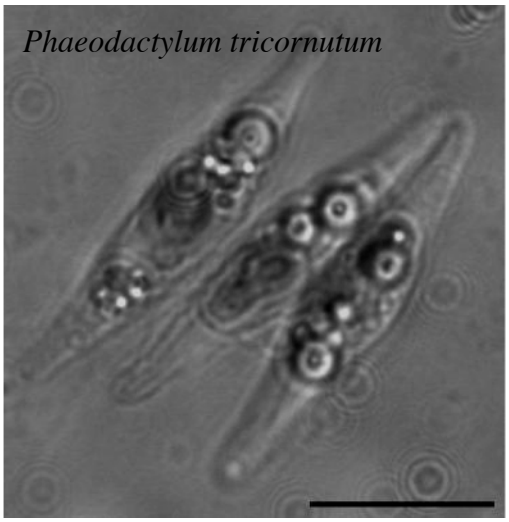
Heterokontophyta (heterokonts)

Bacillariophyceae

Chaetoceros muelleri – high levels of saturated and monounsaturated fatty acids (Hu et al. 2008), high lipid content (33.6% of dry wt.) (Rodolfi et al. 2009).

Cyclotella cryptica – model alga for genetic manipulation studies of lipid synthesis (Hu et al. 2008).

Phaeodactylum tricornutum – marine alga (Rodolfi et al. 2009); accumulates high amounts of EPA (Belarbi et al. 2000); high lipid content (18.7% of dry wt.) (Rodolfi et al. 2009).



Eustigmatophyceae

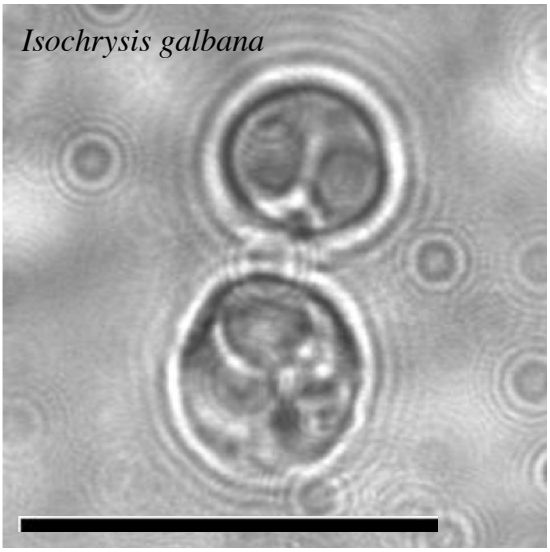
Monodus subterraneus – freshwater alga (Rodolfi et al. 2009); high lipid content (16.1% of dry wt.) (Rodolfi et al. 2009); increased lipid content (39.3% of dry wt.) when phosphate deprived (Khozin-Goldberg and Cohen 2006).

Nannochloropsis sp. – marine alga (Rodolfi et al. 2009); high oil content (31-68% of dry wt.) (Tran et al. 2010); high triacylglycerol content (41.1% of dry wt.) (Wang and Wang 2011); high productivity (20-30 tons/ha/yr) (Scott et al. 2010).

Prymnesiophyta (haptophytes)

Pymnesiophyceae

Isochrysis sp. – marine alga (Rodolfi et al. 2009); high oil content (22.4% of dry wt.) (Rodolfi et al. 2009); high triacylglycerol content (26.0% of dry wt.) in nutrient deficient conditions (Huber et al. 2006); high PUFA producer (Renaud et al. 1995).


Isochrysis galbana

Sagenista (fungoid protist)*

Labyrithuleae

Schizochytrium sp. – accumulates high amounts of DHA (20% of dry wt.) (Harwood and Guschina 2009).

* *Schizochytrium* have been classified variously as either a fungus or a protist, though recently have been included in microalgae.

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